Growth and metabolism in adult *Drosophila*

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DECLARATION OF AUTHENTICITY

The research reported in this thesis was carried out in the Division of Physiology and Metabolism at the MRC National Institute for Medical Research (Mill Hill, London), under the supervision of Dr Alex Gould.

I, Panayotis Pachnis, declare that the work presented in this thesis is the result of my own independent work. Any collaborative work or data provided by others has been indicated within the respective chapters.

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ABSTRACT

In mammals, dietary nutrients regulate growth and metabolism via a network of specialised organs, including the liver and adipose tissue. Nutrient availability is coupled to growth and metabolism via Insulin and amino acid signals transduced by target of Rapamycin (TOR) and phosphatidylinositol 3-kinase (PI3K). Diet also has multiple complex influences upon lipid metabolism, impacting upon fat storage and numerous other processes. It is known that TOR/PI3K signalling and lipid metabolism can be misregulated in human metabolic disorders such as type 2 diabetes but the underlying mechanisms are complex and challenging to dissect. *Drosophila* is emerging as a useful model for studying the integrative physiology of lipid metabolism. However, despite much research on lipid metabolism in the developing larva, relatively little is known in the adult fly about which organs other than the fat body are important. To generate new tools to study this issue, I developed an amino acid-defined holidic diet and identified physiological markers of age-dependent metabolism. Using these, I demonstrated that an adult cell type previously implicated in cuticular hydrocarbon synthesis, the adult oenocyte, accumulates lipid droplets in response to a dietary deficiency in methionine. This response is similar to that described for the liver and requires Lpr1, an orthologue of the mammalian Low Density Lipoprotein Receptor. Although many tissues do not grow during adulthood, oenocytes undergo a surprising amino acid and TOR/PI3K dependent volume increase without nuclear DNA replication. Cell ablations and tissue-specific genetic manipulations support a model in which there is bidirectional lipid metabolic cooperation between adult oenocytes and fat body. Together, these results reveal that adult oenocytes are nutrient-responsive and play a hitherto unknown role in lipid metabolism. They also indicate that there are interesting parallels between the metabolic regulation of hepatocytes and adult oenocytes.

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ABBREVIATIONS

ACAT	Acyl-CoA cholesterol transferase
ACC	Acetyl-CoA carboxylase
ACLY	ATP-citrate lyase
ACP	Acyl carrier protein
AKH	Adipokinetic hormone
ATGL	Adipose triglyceride lipase
CBS	Cystathionine β-synthase
CC	Corpora Cardiaca
CDD	Chemically defined diet
CDP	Cytidine diphosphate
Cpr	Cytochrome P450 reductase
CPT1	Carnitine:palmitoyltransferase 1
Cyp4g1	Cytochrome P450 4G1
DAG	Diacylglycerol
DGAT	Diacylglycerol acyltransferase
DHA	Docosahexaenoic acid
DHCA	Dihydroxycholestanoic acid
DILPs	*Drosophila* Insulin-like peptides
DPA	Docosapentaenoic acid
DR	Dietary restriction
EdU	5-ethynyl-2′-deoxyuridine
FA	Fatty acid
FAS	Fatty acid synthase
FB	Fat body
FFA	Free fatty acid
GAMT	Guanidinoacetate *N*-methyltransferase
GC-MS	Gas chromatography mass spectrometry
GNMT	Glycine *N*-methyltransferase
HCC	Hepatocellular carcinoma
HDL	High density lipoprotein
Hnf4	Hepatocyte nuclear factor 4

HSL	Hormone sensitive lipase
InR	Insulin receptor
LCFA	Long chain fatty acid
LDL	Low density lipoprotein
LDLR	Low density lipoprotein receptor
Lpp	Lipophorin
LpR	Lipophorin receptor
mNSC	Median neurosecretory cell
MTP	Microsomal triacylglycerol transfer protein
MUFA	Mono-unsaturated fatty acid
NAFLD	Non-alcoholic fatty liver disease
NASH	Non-alcoholic steatohepatitis
NEFA	Non-esterified fatty acid
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
PEMT	Phosphatidylethanolamine *N*-methyltransferase
PKA	Protein kinase A
PUFA	Poly-unsaturated fatty acid
RNAi	RNA interference
ROS	Reactive oxygen species
SAH	S-adenosylhomocysteine
SAM	S-adenosylmethionine
SAM-S	S-adenosylmethionine synthetase
SCD	Stearoyl-CoA desaturase
SREBP	Sterol regulatory element-binding protein
TAG	Triacylglycerol
THCA	Trihydroxycholestanoic acid
TOR	Target of Rapamycin
TSP	Trans-sulfuration pathway
VLCFA	Very long chain fatty acid
VLDL	Very low density lipoprotein
WPP	White prepupa

CHAPTER ONE

Introduction

CHAPTER ONE: Introduction

1.1 Scope of the present work

In this introduction I will describe the ways in which organisms regulate lipid metabolism to meet fluctuating energy demands, and the central role that the liver plays in this processes. The influence of nutrition on metabolism will also be introduced, including recent studies on *Drosophila*, the experimental model used in this thesis. The adipose axis of *Drosophila* will be introduced, with a focus on adult oenocytes, the main subject of my investigations. I will introduce their known role in hydrocarbon synthesis and how I aim to identify new and more general functions for oenocytes in lipid metabolism.

1.2 Lipid metabolism overview

Lipids constitute a large class of hydrophobic molecules that play important structural and signalling roles, as well as providing an efficient form of energy storage (reviewed in Gurr et al., 2002). Many of these functions are carried out by fatty acids, which also serve to integrate lipid with amino acid/carbohydrate metabolism (Frayn et al., 2006). Fatty acids can be synthesised *de novo* or ingested from the diet, after which they can be further modified by elongase and desaturase enzymes, respectively lengthening the fatty acid carbon chain and introducing C=C double bonds. Depending upon the number of double bonds, fatty acids can be classified into saturated, monounsaturated (MUFA) or polyunsaturated fatty acids (PUFA). Fatty acids can be subsequently incorporated into more complex lipids, such as glycerolipids, sphingolipids and cholesterol esters. These lipid species can also be readily broken down again by lipases to release free fatty acids for further metabolism or oxidation (Young and Zechner, 2013). In mammals a complex lipoprotein system has evolved to transport hydrophobic lipids between organs according to their metabolic needs (Lee et al., 2003).

1.2.1 *De novo* fatty acid synthesis

De novo fatty acid biosynthesis is a multi-step process in which 2 carbon units are sequentially added to acetyl-CoA to yield 16 and 18 carbon products (reviewed in Strable and Ntambi, 2010). The initial step of this reaction is regulated by acetyl-CoA carboxylase (ACC) and involves the enzymatic conversion of acetyl-CoA (derived from the TCA cycle) to malonyl-CoA (Kim, 1997). This is then followed by the replacement of coenzyme A with acyl carrier protein (ACP), a small molecular mass protein. FA elongation occurs by cycling through a four-step process (condensation, reduction, dehydration and reduction). In the first step, acyl-ACP is condensed with malonyl-ACP to produce 3-ketoacyl-ACP, in a reaction catalysed by 3-ketoacyl-ACP synthase. In the second step, NADPH is used to reduce 3-ketoacyl-ACP to 3-hydroxyacyl-ACP via a 3-ketoacyl-ACP reductase. 3-hydroxyacyl-ACP is subsequently dehydrated by 3-hydroxyacyl-ACP dehydrase, generating enoyl-ACP. Finally, enoyl-ACP is reduced to an acyl-ACP (containing two more carbon units than the original acyl-ACP). This step requires NADPH and is catalysed by enoyl-ACP reductase. This group of 4 enzymatic activities is collectively known as fatty acid synthase (FAS). In mammals they encoded by a single gene, *FASN*, and form a multifunctional enzyme where each partial reaction is catalysed by a discrete domain.

Stearoyl-CoA desaturase (SCD, a Δ9 desaturase) is the rate limiting enzyme involved in the conversion of C16:0 and C18:0 (products of FAS or derived from the diet) to the MUFAs C16:1 and C18:1, respectively (Flowers and Ntambi, 2008; Ntambi and Miyazaki, 2004). MUFAs act as important signalling molecules and are also the predominant acyl species found in fat storage depots, in the form of the neutral lipids TAG and cholesteryl ester (Cao et al., 2008; Ntambi and Miyazaki, 2004). The very low levels of hepatic cholesteryl esters and triglycerides found in mice homozygous for a mutation in the gene for SCD1 ($SCD^{-/-}$) demonstrate the importance of MUFAs in stimulating the synthesis of these lipid fractions (Miyazaki et al., 2000).

1.2.2 VLCFA synthesis

Very long chain fatty acids (VLCFAs) play key roles in multiple biological processes, including skin barrier formation, retinal function, resolution of inflammation, maintenance of myelin, sperm development and liver homeostasis (Kihara, 2012). The C16 fatty acid precursors of VLCFAs can be either synthesised *de novo* or can be obtained from the diet, except for very long chain-polyunsaturated fatty acids, whose precursors are linoleic acid (C18:2, ω6) and α-linolenic acid (C18:3, ω3), which can only be derived from the diet. This is because mammals lack the Δ12 and Δ15 desaturases required for their synthesis (Lands, 1992; Nakamura and Nara, 2004). VLCFAs are synthesised through a series of elongation, desaturation and β-oxidation reactions. β-oxidation enzymes within peroxisomes are required for the final step in the synthesis of the ω6 and ω3 fatty acids, docosapentaenoic acid (DPA) and docosahexaenoic acid (DHA) respectively (Ferdinandusse et al., 2001).

Following their conversion to acyl-CoAs, fatty acids are elongated by enzymes embedded in the endoplasmic reticulum. As in *de novo* fatty acid synthesis, fatty acid elongation occurs by cycling through a four step process of condensation, reduction, dehydration and reduction (Kihara, 2012). The first condensation step is catalysed by a fatty acid elongase. Mammals have seven elongases, each exhibiting characteristic substrate specificity (Guillou et al., 2010; Jakobsson et al., 2006; Kihara, 2012). The product of this reaction, 3-ketoacyl-CoA, is reduced (using NADPH) to 3-hydroxyacyl-CoA by a 3-ketoacyl-CoA reductase (KAR in mammals, Moon and Horton, 2003). 3-hydroxyacyl-CoA is subsequently dehydrated by 3-hydroxyacyl-CoA dehydratase, generating 2,3-*trans*-enoyl-CoA. Finally, 2,3-*trans*-enoyl-CoA reductase catalyses the NADPH-dependent reduction of 2,3-*trans*-enoyl-CoA to acyl-CoA.

1.2.3 Triacylglycerol synthesis

Fatty acyl-CoA thiolesters can be transferred to glycerol in a stepwise manner to form triacylglycerols (TAGs) in what is known as the glycerol phosphate pathway (Cao et al., 2012). The enzyme that transfers acyl groups to position 1 (acyl-CoA:glycerol phosphate 1-O-acyltransferase, GPAT) exhibits marked specificity for saturated acyl-CoA thiolesters whereas the second enzyme (acyl-CoA:1-acyl glycerol phosphate 2-O-acyltransferase, LPAT) shows specificity towards mono- and dienoic fatty acyl-CoA thiolesters (Gurr et al., 2002). The product of these two reactions, 1,2-diacyl-sn-glycerol 3-phosphate, can be dephosphorylated (by phosphatidate phosphohydrolase, PAP) to yield 1,2-diacyl-sn-glycerol, or it can enter the glycerolphospholipid biosynthetic pathway. The final step of TAG synthesis is catalysed by diacylglycerol acyltransferase (DGAT) and involves the transfer of the final acyl group to 1,2-diacyl-sn-glycerol. Both DGAT and ACAT (acyl CoA cholesterol transferase, responsible for cholesteryl ester synthesis) are found in the endoplasmic reticulum, facilitating incorporation of TAGs and cholesteryl esters into lipid droplets (Brasaemle, 2007). The glycerol backbone of TAG can also be provided by the dihydroacetone (glycerone) phosphate pathway, in which glycerone phosphate, a product of glycolysis, is acylated and then reduced before linking into the main TAG biosynthetic pathway. However, the primary role of the dihydroacetone phosphate pathway is in the formation of ether (alkyl) lipids, alkyl groups bonded to glycerol via an ether linkage. An important example from this lipid class is platelet activating factor (1-O-alkyl-2-acteyl-*sn*-glycero-3-phosphocholine) which causes platelets to release vasoactive amines. The enzymes responsible for catalysing the initial steps of ether lipid synthesis are found in peroxisomes, explaining why patients with peroxisome biogenesis disorders often have reduced levels of these lipids (Steinberg et al., 2006).

1.2.4 Lipid droplets and lipases

Lipid droplets are intracellular organelles, characterised by a neutral lipid core (TAG and cholesteryl esters) and a surrounding phospholipid monolayer, into which numerous proteins are embedded, including several lipases (Brasaemle, 2007; Olofsson et al., 2009; Thiele and Spandl, 2008). Lipid droplets perform important roles in the storage of energy, the regulation of cellular lipid homeostasis and in the production of signalling lipids. As previously mentioned, the neutral lipids present in lipid droplets are synthesised in the endoplasmic reticulum. These neutral lipids accumulate between the luminal and cytoplasmic leaflets of the phospholipid bilayer, before budding off to become distinct structures (Brasaemle and Wolins, 2012). The breakdown of TAG and cholesteryl esters to release fatty acids and cholesterol is mediated by several cytosolic lipases, including adipose triglyceride lipase (ATGL), important for basal levels of lipolysis, and hormone sensitive lipase (HSL), which can be stimulated by catecholamines to increase the rate of lipolysis (Ahmadian et al., 2009; Brasaemle, 2007; Zechner et al., 2012). β-adrenergic receptor activation mediated signalling through G proteins stimulates an increase in cAMP levels and protein kinase A (PKA) activation, which in turn phosphorylates HSL (Brasaemle, 2007; Zechner et al., 2012). Phosphorylation of HSL activates lipase activity and, more importantly, triggers the translocation of HSL from the cytoplasm to lipid droplets where it can act upon neutral lipids (Brasaemle, 2007; Zechner et al., 2012). This translocation is thought to mediated, at least in part, by the interaction between HSL and perilipin, a member of the evolutionarily conserved PAT family of proteins, which is itself a substrate for PKA (Bickel et al., 2009; Brasaemle, 2007; Greenberg et al., 2011; Olofsson et al., 2009). Perilipin reduces basal lipolysis by protecting TAG from cytosolic lipases, intriguingly however, it is also required for maximal stimulated lipolysis catalysed by HSL. By regulating the rate of lipolysis, perilipin allows the cell to rapidly respond to fluctuating metabolic demands placed upon it.

Cells are also able to break down lipids through a novel autophagic pathway termed lipophagy (Singh and Cuervo, 2012; Singh et al., 2009). During this process, an autophagosome is formed around part of a lipid droplet, before it fuses with lysosomes containing acid lipases that are able to hydrolyse the sequestered lipids (Singh and Cuervo, 2012; Singh et al., 2009). Lipophagy is believed to act in parallel with cyotosolic lipases during the rapid and efficient breakdown of lipids that occurs during processes such as starvation.

1.2.5 Fatty acid β-oxidation

Fatty acids are primarily catabolized by the β-oxidation pathway, which is composed of a cyclical series of reactions that result in the shortening of fatty acids by two carbons per cycle (Wanders et al., 2010b). The first step in this process involves the transport of fatty acids into mitochondria, via the carnitine shuttle, where the enzymes of β-oxidation are expressed. Fatty acyl-CoAs cannot directly enter the mitochondria, necessitating their conversion to acylcarnitines through the activity of carnitine:palmitoyltransferase 1 (CPT1), that is located on the outer mitochondrial membrane. Acylcarnitines cross the outer mitochondrial membrane via porin, and the inner mitochondrial membrane via carnitine:acylcarnitine translocase, before being converted back to acyl-CoAs by CPT2, located on the inner mitochondrial membrane. Rates of β-oxidation are tightly regulated by the activity of CPT_1, which is strongly inhibited by ACC-dependent malonyl-CoA production. This tends to ensure that β-oxidation is low when lipogenesis is high and *vice versa*. For mitochondrial β-oxidation two carbon atoms are removed from the fatty acyl chain in a sequence of steps involving oxidation, hydration, oxidation and thiolysis, catalysed by acyl-CoA dehydrogenase, enoyl-CoA hydratase, L-3-hydroxyacyl-CoA dehydrogenase and 3-ketoacyl thiolase, respectively. In each round of oxidation, NADH, $FADH_2$ and acetyl-CoA are generated until the last cycle when two acetyl-CoA molecules are produced from the catabolism of a four-carbon fatty acid. NADH and $FADH_2$ can then enter the

electron transport chain to produce ATP. Acetyl-CoA can either enter the Krebs cycle for ATP production or it can be oxidised in the cytosol, generating NADPH that has important roles in maintaining redox balance and in powering fatty acid synthesis. Recent studies have revealed that in the context of cancer metabolism, ATP and NADPH generated through fatty acid oxidation are essential for cells to counteract metabolic stress, raising the possibility of novel therapeutic targets (Carracedo et al., 2013).

Peroxisomes are also an important site of β-oxidation, specialised in the catabolism of LCFAs and VLCFAs to medium chain fatty acids (Wanders et al., 2010a). Because peroxisomes lack the appropriate enzymatic machinery for the complete breakdown of fatty acids to acetyl-CoA, medium-chain fatty acid products have to be transported from peroxisomes in a carnitine acyltransferase-dependent manner to the mitochondria for further oxidation. Unlike mitochondrial β-oxidation, the primary step of peroxisomal β-oxidation is catalysed by acyl-CoA oxidase, producing H_2O_2 as a by-product which can be subsequently detoxified by conversion to hydrogen and water by peroxisome-specific catalase (Wanders et al., 2010a). Finally, peroxisomal β-oxidation is also required for the metabolism of the bile acid synthesis intermediates dihydroxycholestanoic acid (DHCA) and trihydroxycholestanoic acid (THCA) (Wanders et al., 2010a).

1.2.6 Fatty acid α-oxidation

α-oxidation, active in peroxisomes, is required for the breakdown of 3-methyl branched-chain fatty acids that are structurally unable to undergo β-oxidation as a result of the methyl group at the 3-position (Wanders et al., 2010a). An important example of a 3-methyl fatty acid is the plant-derived phytanic acid, whose breakdown starts with the formation of the CoA-ester (Wanders et al., 2011). Subsequently, hydroxylation of phytanoyl-CoA to 2-hydroxyphytanoyl-CoA is catalysed by phytanoyl-CoA hydroxylase. This is followed by cleavage of 2-hydroxyphytanoyl-CoA to formyl-CoA and pristanal, which is then oxidised to pristanic acid. After this point, pristanic

acid can be activated to pristanoyl-CoA which can undergo beta oxidation as described above.

1.2.7 Lipids are transported as lipoproteins

Lipids, being insoluble in water, need to be combined with proteins for transport in the blood. Free fatty acids (FFA) are toxic in the unbound form and so are maintained at low concentrations in the plasma by binding to serum proteins such as albumin (Olson, 1998). A separate mechanism has evolved whereby protein moieties, known as apolipoproteins, interact with neutral lipids (particularly TAG and cholesteryl esters) to form lipoprotein particles that can be classified according to size, density and composition (Lee et al., 2003). In addition to stabilizing lipid particles in the aqueous environment of the blood, apolipoproteins play a critical role in conferring 'identity' to lipoprotein particles. Different apolipoproteins are recognised by tissue-specific receptors, thereby providing an elegant method for selectively targeting lipoprotein particles around the body (Lee et al., 2003).

TAG-rich lipoproteins, termed chylomicrons, are well suited to the transport of exogenous (dietary) lipids from enterocytes into the systemic circulation, whereas very low density lipoproteins (VLDLs) perform a crucial role in the transport of TAG synthesized in the liver (Gibbons et al., 2004; Mansbach and Siddiqi, 2010). VLDL particles are formed around a molecule of apolipoprotein B100, to which TAG is added by a process involving the microsomal triacylglycerol transfer protein (MTP) (Hussain et al., 2008). Both chylomicrons and VLDL are acted upon by extracellular lipoprotein lipases to release fatty acids which can be utilized by various extra-hepatic tissues, including adipose tissue, skeletal and cardiac muscle, and mammary gland. Chylomicrons and VLDL lipoproteins that have been modified by lipoprotein lipases form chylomicron remnants and low density lipoprotein (LDL) particles, respectively (Gibbons et al., 2004; Mansbach and Siddiqi, 2010). LDLs, rich in cholesterol, phospholipids and proteins, are the major carriers of plasma cholesterol to peripheral tissues (in a process known as forward transport) for steroidogenesis and maintenance of cell

membrane integrity (Lee et al., 2003; Willnow, 1999). Both chylomicron remnants and LDL particles are eventually taken up by hepatocytes via lipoprotein receptors, as a first step in their subsequent catabolism (Lee et al., 2003; Willnow, 1999). A further class of lipoprotein particle, high density lipoprotein (HDL), mediates the transfer of cholesterol from the periphery to the liver where it can be further processed or excreted (Francis, 2010). In this pathway (known as reverse transport) the liver is initially responsible for the synthesis of HDL precursor particles (pre-β HDL), which then acquire cholesterol from cells and other lipoprotein particles, resulting in mature HDL particles that are eventually targeted back to the liver (Francis, 2010).

Two mechanisms exist to release the various components of lipoproteins. The first requires the action of extracellular lipases and the subsequent uptake of free fatty acids via specialized transporter molecules in the plasma membrane. The second mechanism involves the endocytosis of lipoproteins, a process mediated by a family of specialised lipoprotein receptors. For example, the well-characterized LDL-receptor is essential for the uptake of lipids into cells and for the regulation of the concentration of cholesterol-rich lipoproteins in the circulation (Goldstein and Brown, 1974). Once internalized, lipoprotein particles enter the lysosomal degradation pathway where the apolipoproteins are broken down into amino acids and lipids are released into the cytosol (Willnow, 1999). In addition to their role in lipoprotein endocytosis, lipoprotein receptors mediate an extensive range of essential biological functions such as signal transduction in neuronal migration, synaptic plasticity and vitamin homeostasis (Nykjaer and Willnow, 2002; Willnow et al., 1999).

1.2.8 SREBP-dependent regulation of lipid metabolism

Sterol regulatory element-binding proteins (SREBPs) are a family of nutritionally responsive transcription factors that regulate lipid metabolism (Horton et al., 2002; Shao and Espenshade, 2012). Mammals have three SREBP isoforms; SREBP-1a and SREBP-1c regulate fatty acid, phospholipid

and TAG biosynthesis, whereas SREBP-2 regulates cholesterol biosynthesis via a well-defined regulatory feedback mechanism (Horton et al., 2002). SREBP-2 is produced as an inactive precursor stored with the INSIG/SCAP chaperone complex in the ER. Depletion of cholesterol promotes release from INSIG and SCAP mediated transit of SREBP-2 to the Golgi, followed by S1P and S2P-mediated proteolytic maturation and nuclear translocation of the transcriptionally active N-terminal portion to stimulate cholesterogenic gene expression (Brown and Goldstein, 1997; Horton et al., 2002). The mechanisms regulating SREBP-1 are less well understood, although it has been shown that insulin signalling can increase its activity through mTORC1-dependent and independent pathways (Porstmann et al., 2008; Yecies et al., 2011). SREBP-1 activates FASN and ATP-citrate lyase (ACLY)-dependent *de novo* lipogenesis and is required for Akt-dependent cell growth (Porstmann et al., 2008). Intriguingly, a recent study (Walker et al., 2011) has uncovered a novel function for the one carbon cycle in the regulation of SREBP-1-mediated lipogenesis, which will be discussed in greater detail in section 1.4.1.

Importantly, insects are unable to synthesise cholesterol and therefore have to obtain it from their diet (Carvalho et al., 2010; Clayton, 1964). Accordingly, *Drosophila* SREBP (dSREBP) does not respond to sterol levels, but instead is regulated by phosphatidylethanolamine (PE), the major phospholipid in *Drosophila* (Lim et al., 2011). A reduction in phosphatidylethanolamine stimulates dSREBP signalling which in turn activates lipogenesis in an ACLY and FASN-dependent manner (Lim et al., 2011).

1.3.1 Liver physiology

The liver is a central site of metabolic integration, where FAs are mobilised and either stored or used as an energy source depending on demand. In the fed state, both *de novo* lipogenesis and TAG synthesis occur in liver hepatocytes (Coleman and Lee, 2004; Gibbons et al., 2000; Hellerstein et al., 1996). Lipids and their metabolites may then be exported from the liver for

utilization by peripheral tissues, such as muscles, or for storage in adipose tissue (Fig. 1.1). Conversely, food deprivation stimulates the lipolysis of TAG stored in mammalian adipocyte fat droplets via increases in Hormone-Sensitive Lipase (HSL) and Adipose Triglyceride Lipase (ATGL) activity (Lass et al., 2011; Zechner et al., 2005). Stimulation of lipolysis results in TAG breakdown and the release of fatty acids into the circulation, which can then be taken up by hepatocytes and β-oxidized into ketone bodies (Fig. 1.1) (McPherson and McEneny, 2012). These water-soluble compounds provide a crucial energy source in the starved state for several organs, especially the brain (Finn and Dice, 2006).

1.3.2 Hepatic steatosis and insulin resistance

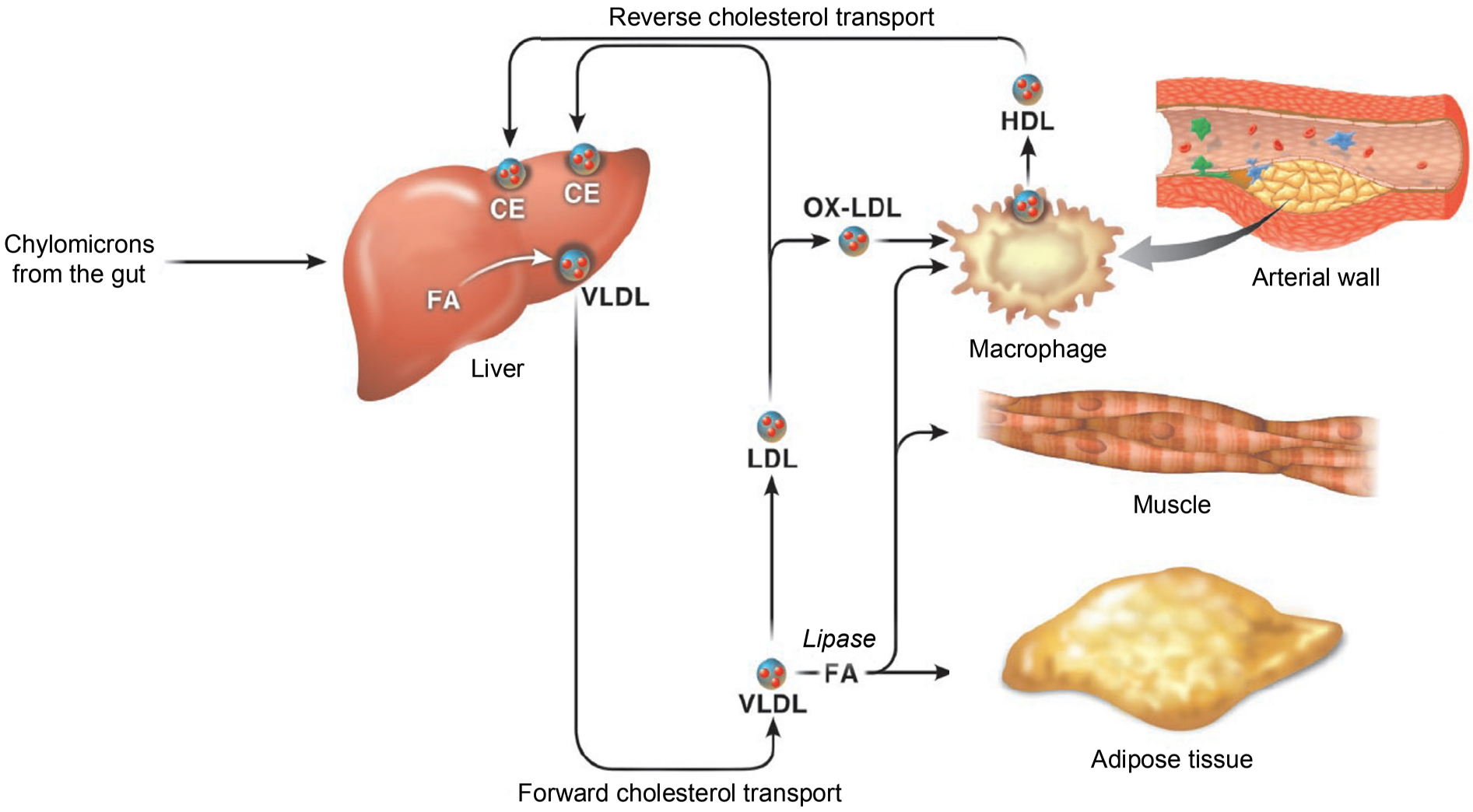
Non-alcoholic fatty liver disease (NAFLD), characterised by the excessive accumulation of lipids in the liver, is one of the most prevalent metabolic disorders (Farese et al., 2012). This term encompasses several grades of pathology, starting from simple steatosis and progressing to the more severe non-alcoholic steatohepatitis (NASH) and cirrhosis. Accumulation of hepatic lipids can result from increased dietary intake, increased adipose lipolysis and release of free fatty acids, increased *de novo* lipogenesis, or decreased hepatic lipolysis and oxidation (Cohen et al., 2011; Sozio et al., 2010). Multiple single-gene mutations have been characterised that perturb the above pathways and result in NAFLD (Cohen et al., 2011). However, there is also a strong dietary component in the aetiology of the disorder, as evidenced by the high prevalence of NAFLD among the severely obese. In these individuals, the increased 'overflow' of free fatty acids to the liver from the diet, from adipose tissue and through increased *de novo* lipogenesis all serve to promote hepatic steatosis (Brookheart et al., 2009; Cohen et al., 2011). However, it is now believed that hepatic TAG accumulation in NAFLD serves as a protective mechanism, possibly by preventing the build-up of toxic substrates such as free fatty acids (Bass, 2010; Neuschwander-Tetri, 2010). In support of this, knockdown of DGAT (catalysing the final step of

TAG synthesis) reduces hepatic steatosis but exacerbates liver damage and fibrosis in obese mice with NASH (Yamaguchi et al., 2007).

Hepatic steatosis is strongly correlated with insulin resistance, however, it is still unclear whether one drives the other or whether they both result from a common underlying molecular pathophysiology (Farese et al., 2012). In liver, insulin inhibits glucose production and promotes fatty acid synthesis. With the development of hepatic insulin resistance, the inhibitory effect of insulin on glucose production is diminished, whereas the stimulatory effect of insulin on lipogenesis is retained, resulting in hepatic TAG accumulation (Brown and Goldstein, 2008). Consequently, interventions that ameliorate insulin resistance lead to lower insulin levels and decreased hepatic TAG content (Cohen et al., 2011). It is still a matter of debate whether steatosis can promote insulin resistance. However, recent studies have demonstrated that mice with genetic defects in lipolytic or TAG synthetic pathways can develop hepatic steatosis without insulin resistance (Brown et al., 2010; Hoy et al., 2011; Minehira et al., 2008; Monetti et al., 2007). This is further supported by studies on humans with genetic variants that promote steatosis but fail to induce insulin resistance (Hooper et al., 2011). Together, these results indicate that hepatic steatosis is not sufficient for insulin resistance.

Figure 1.1 The liver as a central player in lipid metabolism

The liver is an integrative site for lipid homeostasis, where lipids are distributed to peripheral tissues for storage and energy consumption (forward lipid transport) or captured from adipocytes and macrophages to be recycled or excreted (reverse lipid transport). Lipids are transported around the body through several distinct types of lipid/protein complexes termed lipoproteins (Adapted from Lee et al., 2003).


Reverse cholesterol transport
HDL
CE
CE
OX-LDL
Chylomicrons
from the gut
FA
VLDL
Liver
Macrophage
Arterial wall
LDL
Muscle
Lipase
FA
VLDL
Adipose tissue
Forward cholesterol transport

1.4.1 One carbon metabolism

Methionine regulates redox equilibrium, epigenetic modifications and multiple physiological processes such as ageing. It is also well established that diets lacking methionine, often used to generate animal models of NAFLD, promote hepatic steatosis, demonstrating that methionine is a key regulator of lipid metabolism (Basaranoglu et al., 2010; Koteish and Diehl, 2001). Despite extensive research, many questions remain to be answered regarding the mechanisms underlying this regulation.

Methionine is metabolised through a cyclical series of reactions referred to as one carbon metabolism (Mato et al., 2008). Conversion of methionine to S-adenosylmethionine (SAM) is followed by conversion to S-adenosylhomocysteine (SAH), which can in turn be hydrolysed to homocysteine. Homocysteine can be remethylated to generate methionine, catalysed by methionine synthase or betaine-homocysteine methyltransferase, or it can enter the transsulfuration pathway for further metabolism, as discussed below.

SAM is a methyl donor that has important roles in cell growth, apotosis and liver cancer (Lu and Mato, 2008). The liver has a central role in SAM homeostasis, as it is the principle site of its synthesis and degradation. There are multiple SAM-dependent trans-methylation reactions, however, three reactions contribute most to the transmethylation flux:

1. Methylation of glycine by glycine *N*-methyltransferase (GNMT) to form sarcosine.
2. Methylation of guanidinoacetate by guanidinoacetate *N*-methyltransferase (GAMT) to form creatine.
3. Methylation of phosphatidylethanolamine by phosphatidylethanolamine *N*-methyltransferase (PEMT) to form phosphatidylcholine (PC) (Mato et al., 2008).

In the liver, PEMT-dependent PC synthesis accounts for approximately 30% of total hepatic PC, with the rest synthesised via the SAM-independent cytidine-diphosphate-choline (CDP-choline) pathway (Gibellini and Smith, 2010). PC molecules synthesised via the SAM-

dependent pathway contain a greater proportion of polyunsaturated acyl groups. Intriguingly, the expression of the CDP-choline pathway associates with liver proliferation and carcinogenesis, whereas PEMT expression diminishes during liver regeneration and after partial hepatectomy, and is inhibited in hepatocellular carcinoma (HCC) (Mato et al., 2008). Importantly, PEMT-dependent PC synthesis provides a mechanistic link between methionine and aberrant lipid metabolism. Low intracellular SAM concentrations (e.g. by feeding on a methionine deficient diet) inhibit flux through the PEMT-dependent transmethylation pathway, resulting in impaired PC synthesis. As PC is required for the assembly and secretion of VLDL particles, reduced levels will inhibit VLDL-mediated lipid export resulting in hepatic steatosis (Vance, 2008; Yao and Vance, 1988). This is supported by genetic studies in PEMT knockout mice, which exhibit reduced levels of PC and hepatic steatosis (Zhu et al., 2003). Intriguingly, Walker *et al.* have recently shown that reduced SAM and PC levels stimulate lipogenesis via an alternative SREBP-1-dependent mechanism, which is independent of both cholesterol and SCAP (Walker et al., 2011). Instead, the authors found that reduced PC inhibits ARF-GTP-ase signalling, allowing relocalisation of S1P and S2P to the ER and proteolytic activation of SREBP-1 (Walker et al., 2011).

GNMT, the most abundant methyltransferase in mammalian liver, catalyses the conversion of glycine into sarcosine, which is then oxidized to regenerate glycine (Mato et al., 2008). Despite no known metabolic role for sarcosine itself, it is believed that the function of this cycle is to catabolise excess hepatic SAM. In support of this, GNMT knockout mice display elevated hepatic SAM levels, which induce the PEMT-dependent conversion of PE to PC (Martinez-Una et al., 2013). In order to maintain a normal PC/PE ratio, the liver employs two mechanisms to prevent PC levels from becoming exceedingly high. Firstly, the liver stimulates PC secretion via VLDL. Secondly, phospholipase-mediated degradation of PC is stimulated, leading to increased DAG levels which can be rerouted towards TAG synthesis, promoting hepatic steatosis (Martinez-Una et al., 2013).

Dietary studies together with genetic studies on SAM-dependent methyl-transferases demonstrate the physiological importance of homeostatically regulating intracellular hepatic SAM levels (Jacobs et al., 2013; Martinez-Una et al., 2013). It is clear that both depleted and elevated SAM concentrations can promote PEMT-dependent lipid dysregulation. Additional PEMT-independent effects have been observed in GNMT knockout mice with elevated SAM levels, including aberrant DNA and histone methylation, resulting in activation of the JAK/STAT pathway and development of hepatocellular carcinoma (Martinez-Una et al., 2013).

1.4.2 The transsulfuration pathway

The transsulfuration pathway (TSP) directs the conversion of homocysteine to cysteine for protein synthesis, or further metabolism to glutathione, taurine or sulphate (Stipanuk, 1986). Defects in this pathway perturb gluatathione-mediated cellular redox homeostasis and also result in elevated homocysteine levels that have been associated with neurological and cardiovascular degenerative disorders (Beyer et al., 2004; Herrmann and Knapp, 2002; Isobe et al., 2005). Interestingly, patients with a deficiency of cystathionine β-synthase (CBS), responsible for catalysing the first step in the transsulfuration pathway, often develop hepatic steatosis (Aitken and Kirsch, 2005; Gaull et al., 1974). CBS knockout mice also exhibit hepatic steatosis, together with elevated liver and serum TAG and non-esterified fatty acids (NEFA), possibly as a result of reduced β-oxidation (Namekata et al., 2004). Impaired VLDL secretion from the liver as a result of reduced apolipoprotein B100 (apoB100) levels may also contribute. Finally, CBS deficiency was shown to promote oxidative stress, resulting in liver fibrosis and hepatocyte injury (Robert et al., 2005).

A recent study identified key components of the TSP in *Drosophila*, demonstrating that increased flux through this pathway is responsible for mediating the effects of dietary restriction (DR) (Kabil et al., 2011). Furthermore, this study indicates that TSP-mediated reductions in protein translation are not required for DR longevity (Kabil et al., 2011).

Sulphur-containing compounds, including cysteine, taurine and glutathione, regulate cellular redox homeostasis and act to prevent intracellular oxidative damage (reviewed in Metayer et al., 2008; Stipanuk, 1986). Glutathione protects cells from oxidative stress by donating a reducing equivalent to unstable molecules such as reactive oxygen species (ROS). Oxidised glutathione rapidly forms a glutathione dimer, which can subsequently be reduced again by glutathione reductase, or it is eliminated from the cell. The thiol group of glutathione can also interact directly with proteins, forming disulphide glutathiolated proteins, thereby providing an alternative protective mechanism against oxidative damage. Finally, the methionine sulfoxide reductase system reduces ROS-mediated oxidised methionine residues back to methionine, providing a further route for the removal of ROS species.

1.5.1 *Drosophila* as a model organism for the study of lipid metabolism

Recent studies have revealed that many of the regulatory pathways and enzymes involved in lipid metabolism in mammals are conserved in 'lower' organisms such as the fruit fly, *Drosophila melanogaster* (Schlegel and Stainier, 2007). *Drosophila* is a powerful model organism that offers unique genetic tools for the study of a diverse range of physiological and pathophysiological conditions related to lipid metabolism (reviewed in Baker and Thummel, 2007; Leopold and Perrimon, 2007). It is utilized extensively in medical research as ~70% of human disease genes have clear orthologues in the fly genome (Chien et al., 2002; Reiter et al., 2001). In addition to a century of genetics and a fully sequenced genome, several genome-wide resources are now publically available, including larval/adult expression atlases and three RNAi transgene libraries. (Chintapalli et al., 2007; Dietzl et al., 2007; Ni et al., 2008) http://www.shigen.nig.ac.jp/fly/nigfly/index.jsp. A recent genome-wide RNAi obesity screen in adult flies revealed that the Hedgehog pathway in the fat body regulates TAG levels in *Drosophila* (Pospisilik et al., 2010). In the

same study, it was also shown that Hedgehog activation in mammals blocks white but not brown adipocyte differentiation. Genome-wide RNAi screens for lipid droplet regulators in *Drosophila* cells have also been performed (Beller et al., 2008; Guo et al., 2008). This approach identified some factors (e.g. the vesicle-mediated Coat Protein Complex I (COPI) transport complex) that regulate the interaction between lipid droplets and various proteins and lipases (Beller et al., 2008). In addition, proteomic studies have revealed a striking similarity between *Drosophila* and mammalian lipid droplets highlighting that *Drosophila* is a useful model in this area (Beller et al., 2006; Cermelli et al., 2006). Two *Drosophila* PAT proteins, PERILIPIN-1 and -2 (PLIN1 and PLIN2), have been shown to localize to lipid droplets and to regulate lipid metabolism. PLIN2 is involved in promoting lipid accumulation whereas PLIN1 is involved in activating lipolysis and is thought to be the target of various lipolysis stimulating hormones (Arrese et al., 2006; Beller et al., 2010; Gronke et al., 2003; Patel et al., 2005; Teixeira et al., 2003). Chief among these is the adipokinetic hormone (AKH) family of peptides which have important catabolic effects and are thought to be functional analogues of mammalian glucagon (Arrese et al., 2006; Bharucha et al., 2008; Gronke et al., 2007; Patel et al., 2005). It is important to note however, that even within a single cell the protein composition of lipid droplets may vary markedly, possibly reflecting functional differences (Beller et al., 2006; Wilfling et al., 2013). Finally, the transient association of lipid droplets with certain proteins such as histones, reflects a possible role of this organelle as a storage depot for proteins that lack appropriate binding partners in the cell (Cermelli et al., 2006).

Powerful lipidomics tools such as shotgun profiling by high-resolution mass spectroscopy, provide a rapid and comprehensive snapshot of full-lipid composition, accounting for major lipid classes and individual species within each class (Schwudke et al., 2007). These techniques have recently been applied to the study of *Drosophila* metabolism, revealing interesting diet-, developmental- and sex-specific differences in lipid composition (Carvalho et al., 2012; Hammad et al., 2011; Parisi et al., 2011).

Together, the above studies argue that *Drosophila* is well suited for studying lipid metabolism and its powerful tissue-specific genetic tools now allow this to be done at the integrative physiology level (Brand and Perrimon, 1993; Potter et al., 2010). Moreover, exciting results have already emerged from studies utilizing *Drosophila* to model the pathophysiologies underlying diabetes and obesity (Baker and Thummel, 2007; Birse et al., 2010; Rulifson et al., 2002).

1.5.2 The nutritional requirements of *Drosophila melanogaster*

Experiments addressing the link between nutrition and metabolism in *Drosophila* would greatly benefit from the development of a reliable chemically defined diet (CDD). Work in this area is based upon Sang's pioneering studies (Sang, 1956; Sang and King, 1961). Casein (the major milk protein) is used as a protein source in many of the defined diets. However, this does not permit manipulations at the individual amino acid level. To overcome this, defined amino acid mixes have been developed although they generally delay larval growth compared with the optimum casein diet. One possible reason for this is that amino-acid based diets can cause serious osmotic problems for developing larvae (House, 1966). Sugars are a further dietary requirement, with fructose superior to sucrose, which in turn is better than glucose, as judged by pupation rates and adult emergence (Hassett, 1948). Two classes of dietary lipids exist; sterols and fatty acids. The inability of *Drosophila* to synthesise cholesterol makes it an essential component of the diet, although other sterols can be used as substitutes (Cooke and Sang, 1970). Both Sang and Rapport *et al.* did not include fatty acids in their diets, demonstrating that they are a non-essential component of the diet (Rapport et al., 1983; Sang, 1956). Nevertheless, the lipid composition of the diet can influence the balance of different fatty acids in both the larva and adult body (A. Gould pers. Comm. Keith, 1966; Keith, 1967). Moreover, choline, which functions in the synthesis of phospholipids as well as acetylcholine, is an essential component of the larval diet (Geer et al., 1971; Geer and Vovis, 1965). In addition, B-vitamins were all found to be

necessary, whereas vitamin C and the fat-soluble vitamins were not (Sang, 1956). Finally, dietary RNA has been shown to improve larval growth, although this can be substituted by purified inosine and uridine (Schultz et al., 1946). It is important to note that most of the work on *Drosophila* diets has focused on the nutritional requirements of the larva which must support the ~150x growth associated with this phase of development. While only a few studies have focused specifically on the adult (for example, Sang and King, 1961), it is thought that its nutritional demands are less stringent than those of the larva.

1.5.3 *Drosophila* amino acid/Target of Rapamycin (TOR) and PI3-Kinase signalling

In *Drosophila*, complex regulatory networks act to maintain metabolic homeostasis despite fluctuations in nutrient intake. AKH-producing corpora cardiaca (CC) cells in the ring gland act antagonistically to insulin-producing cells (IPCs) to regulate sugar levels in the haemolymph (Kim and Rulifson, 2004). They mediate their control in part through the production of adipokinetic hormone, a polypeptide with glucagon-like functions. The *Drosophila* genome encodes a unique insulin receptor in addition to seven dILPs which are structural and functional orthologs of mammalian Insulin/IGF (Brogiolo et al., 2001; Teleman, 2010). Ablation of the IPCs, in the fly central nervous system (CNS), results in significantly smaller larvae and adults and generates a diabetic phenotype, including markedly raised haemolymph carbohydrate levels (Ikeya et al., 2002; Rulifson et al., 2002). Insulin signaling is mediated via the highly conserved PI3-kinase pathway, which interacts with the amino-acid sensing TOR network (Polak and Hall, 2009; Teleman, 2010). TOR activation by amino acids requires amino acid transporters, the presence of Rheb and a group of small GTPases, the Rag proteins (Colombani et al., 2003; Hietakangas and Cohen, 2009). The conserved TOR pathway in fat-body cells functions as a global nutrient sensor such that, under amino acid restriction, it no longer sends the systemic fat body signal (FBS) promoting Ilp secretion from IPCs and thus

larval growth (Colombani et al., 2003; Geminard et al., 2009). A recent study has identified Unpaired 2 (Upd2) as a secreted signal produced by the fat body in response to sugar and lipids (Rajan and Perrimon, 2012). Upd2 activates JAK/STAT signalling in a population of GABAergic neurons that project onto the IPCs, thereby relieving the inhibitory tone of the GABAergic neurons on the IPCs, resulting in the secretion of DILPs. However, Upd2 does not appear to be regulated by dietary amino acids, so probably does not correspond to the FBS. An amino acid/TOR-dependent fat-body signal also stimulates local Ilp production in CNS glial cells (Chell and Brand, 2010; Sousa-Nunes et al., 2011). In turn, this regulates early larval neural stem cells (neuroblasts) to exit quiescence. At later stages of larval development, however, neural stem cell growth becomes independent of amino acids in the diet and no longer requires TOR activity (Cheng et al., 2011). At the cellular level, TOR and PI3-Kinase orchestrate a myriad of processes including translation initiation and elongation, ribosome biogenesis and autophagy (Hietakangas and Cohen, 2009; Teleman, 2010).

1.5.4 Nutritional regulation of *Drosophila* fat content, lifespan and heart function

Both 'obese' (e.g. *brummer* mutant) and 'lean' (e.g. *midway* mutant) flies, as defined by their TAG content, can be generated by genetically manipulating ATGL or DGAT1 respectively (Buszczak et al., 2002; Gronke et al., 2005). Recent work has also identified dietary manipulations that alter the TAG content of adult wild-type flies (Skorupa et al., 2008). Nutritional intake is well known to influence a wide range of physiological processes, including ageing and metabolism. When the food intake of organisms is reduced (dietary restriction (DR)), they live longer than organisms fed a normal diet; a phenomenon conserved across diverse species including yeast, flies and mammals (Fontana et al., 2010; Piper and Bartke, 2008). In flies, DR is usually achieved using *ad libitum* food dilution protocols rather than by restricting access to food. Recent work has shown that calorie intake in *Drosophila* is not the only key factor in the reduction of mortality rate by

dietary restriction (Lee et al., 2008; Mair et al., 2005; Skorupa et al., 2008). Yeast, the major source of fly protein, has a much greater effect on lifespan per calorie than sugar (Mair et al., 2005). Follow-up work has shown that imbalances at the individual amino-acid level are responsible for changes in both fecundity and lifespan, suggesting that the ratio of different components within the diet is a critical regulator of various metabolic processes (Grandison et al., 2009). In this study it was demonstrated that adding all non-essential amino acids to a DR diet only marginally shortened lifespan and did not change fecundity, whereas adding all essential amino acids decreased lifespan and increased egg production. Individual amino acid manipulations showed that methionine alone increased fecundity as much as full feeding but without reducing lifespan. In addition, adding all essential amino acids except methionine failed to reduce lifespan, suggesting that methionine, together with one or several other essential amino acids, is responsible for the life-span shortening effect of full feeding (Grandison et al., 2009). In mice, branched-chain amino acid enriched diets have been shown to extend lifespan (D'Antona et al., 2010). It is thought that increased mitochondrial biogenesis and Sirtuin 1 expression in cardiac and skeletal muscle are responsible for this effect, in addition to up-regulation of the reactive oxygen species (ROS) defence system. The effects of branched-chain amino acid enriched diets are abrogated in endothelial nitric oxide synthase (eNOS) null mutant mice, pointing to a key molecular pathway involved in the transduction of nutritional signals (D'Antona et al., 2010). The effects of branched chain versus non-branched chain amino-acids have yet to be explored systematically in *Drosophila*.

In addition to studying the relationship between diet and lifespan, *Drosophila* can also be used to model the genetic mechanisms that link high fat diets with obesity and cardiac dysfunctions. A diet rich in saturated fats tends to lead to cardiac lipid accumulation, reduced cardiac contractility, conduction blocks and severe structural pathologies, reminiscent of human cardiomyopathies (Birse et al., 2010). In this study, systemic inhibition of the TOR pathway prevented excess fat accumulation and protected the *Drosophila* heart against dietary fat induced cardiac dysfunction. It is likely

that this effect is mediated by SREBP1, a key regulator of fat synthesis, as it has been shown that rapamycin inhibition of TOR activity blocks SREBP nuclear accumulation and lipogenesis in flies and in mammals (Lim et al., 2011; Porstmann et al., 2008).

1.6.1 Origins of larval and adult fat body in *Drosophila*

Holometabolous insects such as *Drosophila* possess morphologically different fat-body cell populations during larval and adult (imaginal) stages. This raises the possibility that there may also be functional differences between these two fat-body cell types. The larval fat body, derived from embryonic mesoderm, is composed of an epithelial sheet of white, opalescent polyploid cells located in the body cavity and exposed to the haemolymph (Hoshizaki et al., 1994; Nelliot et al., 2006). During metamorphosis, larval fat-body dissociates into single free-floating cells that persist for the first three days of adulthood, after which they are removed by programmed cell death (Nelliot et al., 2006). The precise developmental origin of the adult fat body is still unclear. Initial suggestions that at least part of it may be ectodermal were overturned by mosaic analysis experiments indicating a mesodermal origin (Lawrence and Johnston, 1986). Gas Chromatography (GC) measurements of TAG indicate that adult fat-body stores approximately 3 times less lipid than does larval fat-body (I. Stefana and A. Gould pers. comm.). Adult fat cells do not fully differentiate until 3-4 days post-eclosion, suggesting that larval fat cells may play an important transitional role in the young adult fly. Consistent with this hypothesis, experimental studies have shown that young adults are nearly three times more resistant to starvation than older adults (Aguila et al., 2007). Furthermore, newly eclosed unfed adults were found to be even more starvation resistant when genetic tools were employed to block larval fat cell death (Aguila et al., 2007). Moreover, over half of the carbon molecules acquired by the ovaries of 2-day adult females are dependent upon the apoptosis of larval fat body (Aguila et al., 2013). Together, these studies suggest that lipids or other molecules stored in the fat body during

larval stages make a substantial contribution to the energy reserves of the young adult fly.

1.6.2 Structural and functional specializations within the fat body of insects

Historically, insect fat body has been considered a single tissue with multiple diverse metabolic functions (Butterworth et al., 1965). It is becoming clear, however, that, like human adipose tissue, the fat body is comprised of discrete structural and functional specializations. Studies on the larval fat body of the corn earworm (*Helicoverpa zea*) have demonstrated that differences exist between peripheral fat body (present from early larval stages) and perivisceral fat body (which appears in the last larval instar) (Haunerland et al., 1990). The former fat body subtype is more involved in protein biosynthesis and the latter more important for protein storage. Furthermore, six regions of the fat body along the anterior-posterior axis were recognized in *Drosophila melanogaster* larvae based on different morphologies (Haunerland and Shirk, 1995). These regions were subsequently shown to be distinct in their ultrastructure, biochemistry, and gene expression pattern. Biochemical differences can be observed under UV light due to the auto-fluorescent properties of certain metabolic intermediates stored in the fat body. For example, kynurenine, a precursor of xanthoommatin (a major screening pigment in the compound eye of *Drosophila*), is stored in the anterior three segments of the larval fat-body (Haunerland and Shirk, 1995). Conversely, the pteridins, a second family of screening pigments are formed and stored only in the fat body of the posterior three segments (Rizki and Rizki, 1962). Work by Hwangbo *et al.* has suggested that functionally distinct fat body subsets relevant to ageing exist in the adult fly (Hwangbo et al., 2004). In this study, over-expression of activated (unphosphorylated) dFOXO in the pericerebral fat of the head was sufficient to increase median lifespan and resistance to oxidative stress. Surprisingly, however, lipid accumulation was observed in this experiment in both the pericerebral and abdominal fat-body.

1.6.3 Insect fat body plays a central role in the regulation of nutrition and metabolism

In the fed state, glycogen and TAG are stored in the insect fat body, an organ that shares similarities with mammalian adipose and hepatic tissue (Butterworth et al., 1965; Law and Wells, 1989). Glycogen is predominantly broken down to release trehalose (composed of two glucose molecules), the main circulating carbohydrate in the insect haemolymph. Fat-body TAG is acted upon by intracellular lipases including Brummer, an ortholog of mammalian Adipose Triglyceride Lipase, and released into the haemolymph primarily as diacylglyceride (DAG)-rich lipophorin particles rather than primarily as free fatty acids (Arrese et al., 2006; Gronke et al., 2005; Gronke et al., 2007). In addition to its storage capabilities, the fat body is responsible for synthesizing many of the major haemolymph proteins, including the apolipoproteins required for lipophorin transport between organs; apolipophorin (apoLpp), apo Lipid Transfer Particle (apoLTP) and crossveinless d (cv-d) (Arrese and Soulages, 2010; Canavoso et al., 2001; Palm et al., 2012). Microsomal triglyceride transfer protein (MTP) is also expressed in the fat body and is required for the processing and release of Lpp and LTP (Palm et al., 2012). Insect lipophorin, similar to mammalian apoB-containing lipoproteins, is associated with two apolipophorin molecules (apolipophorin I and apolipophorin II), both derived from a common proapolipophorin precursor protein encoded by the Lipophorin (Lpp) gene (Canavoso et al., 2001; Panakova et al., 2005). Lipophorin carries the majority of lipids in circulation (Palm et al., 2012). The neutral lipid most commonly carried by lipophorin is *sn*- 1,2 -diacylgycerol, although this varies with physiological status and across insect species (Canavoso et al., 2001). The most abundant phospholipid species in lipophorin particles is phosphatidylethanolamine (Palm et al., 2012). Insect lipophorin particles are able to transport a wide variety of lipids from the fat body and deliver them to specific tissues (Canavoso et al., 2001; Palm et al., 2012). However, lipophorin particles are also able to pick up lipids from the gut in an LTP-dependent manner, for subsequent transport to the rest of the fly (Palm et

al., 2012). In contrast to mammals, lipids in insects are delivered to cells without internalization and destruction of the apolipophorin carrier. This observation led to the idea that apo-lipophorin functions as a reusable shuttle moving lipids from sites of absorption and storage to sites of utilization (Arrese et al., 2001). Two other major differences exist between human and insect lipid metabolism. First, the rate of lipophorin biosynthesis is independent of the amount of lipid in the diet. Instead, there is a direct correlation between the lipid content of lipophorin and the amount of lipid in the diet (Fernando-Warnakulasuriya et al., 1988). Second, insects lack the capacity for *de novo* biosynthesis of cholesterol (Carvalho et al., 2010; Svoboda et al., 1975).

1.6.4 Insect larval oenocytes are hepatocyte-like cells

Oenocytes are an insect cell type that has been recognized for over a century on morphological criteria but without any clearly attributed functions (Gould et al., 2001; Landois, 1865). Like the fat body, there are distinct larval and adult specific populations of oenocytes in *Drosophila*. Larval oenocytes are derived from the dorsal embryonic ectoderm of abdominal segments A1-A7 (Elstob et al., 2001). More recently, at least some of the functions of larval oenocytes were discovered to involve lipid metabolic pathways known to be active in mammalian hepatocytes and other fat-handling cell types (Gutierrez et al., 2007). Using Oil Red O, a neutral lipid stain (Lillie, 1944), it was found that larval oenocytes are unique in specifically accumulating lipids upon starvation, as are mammalian hepatocytes (Gutierrez et al., 2007). This finding has recently been corroborated using coherent anti-Stokes Raman scattering microscopy (Chien et al., 2012). Clonal knockdown experiments show that LpR2 is required in an oenocyte-autonomous manner for starvation-induced steatosis (Parvy et al., 2012). Lipid accumulation upon starvation, together with the observation that larval oenocytes express more than 20 genes encoding orthologues of human hepatic lipid-metabolizing proteins, suggested that larval oenocytes share aspects of regulation with mammalian

hepatocytes, and possibly even functions. Amino-acid/TOR and PI3K signaling in the fat-body regulates the release of fatty acids which may subsequently be taken up by oenocytes. Larval ablation experiments also revealed that oenocyteless (oe^-) larvae display defective molting, do not reach pupariation and show reduced feeding and growth rates, directly or indirectly implicating oenocytes in the regulation of growth and development (Gutierrez et al., 2007).

Ablation of oenocytes results in reduced TAG mobilization from the fat-body upon starvation, suggesting that bidirectional lipid-metabolic coupling exists between the fat body and oenocytes (Gutierrez et al., 2007). However, a recent study has revealed that larval oenocytes also have a role in maintaining the integrity of the tracheal system (Parvy et al., 2012). The authors show that knockdown of ACC in larval oenocytes results in lethality at the L2/L3 transition, as observed upon oenocyte ablation. These larvae display tracheal defects as indicated by the accumulation of liquid in the tracheal trunks, resulting in hypoxia or even anoxia. This effect most likely stems from defects in the spiracles (spiracular ducts and glands), including an absence of neutral lipids that are normally observed in wild-type larvae. Intriguingly, knockdown of KAR, involved in VLCFA synthesis, results in the same phenotypes. Thus the reduced TAG mobilization from the fat body could be an indirect result of hypoxia. Moreover, these results argue that oenocytes synthesise a VLCFA-dependent remote signal that promotes lipid droplet cluster formation in the spiracles, which in turn serves to maintain integrity of the tracheal system and prevent liquid accumulation. From the complex oenocyteless ablation phenotype, it is likely that additional functions for larval oenocytes remain to be discovered.

1.6.5 Adult oenocytes

In *Drosophila,* adult oenocytes have a distinct developmental origin to their larval counterparts, most likely deriving from pupal histoblasts (Fig. 1.2) (Lawrence and Johnston, 1982). Currently, the molecular mechanisms underlying their formation are unknown. Whereas larval oenocytes regulate

TAG accumulation and waterproofing of the tracheal system, adult oenocytes have been shown to synthesise cuticular hydrocarbons mediating desiccation resistance and courtship and mating behaviours (Billeter et al., 2009; Ferveur, 2005; Ferveur et al., 1997; Qiu et al., 2012). Adult oenocyte-ablated (oe^-) flies exhibited a loss of all cuticular hydrocarbons, resulting in dramatic effects on sexual attractiveness (Billeter et al., 2009). Oe^- male flies displayed normal courtship behaviour towards wild-type females, whereas wild-type females were less receptive to oe^- males than control males. Furthermore, oe^- males elicited courtship and copulation attempts from wild-type males. This indicates that oe^- males are perceived as females. Finally, oe^- *Drosophila melanogaster* females mate with wild-type *Drosophila simulans* males, indicating that adult oenocytes are required for the normal pheromone-dependent inhibition to interspecies courtship and copulation (Billeter et al., 2009). Together, these results indicate that oenocytes are required for the cuticular hydrocarbons that confer sex and species specificity.

Insect hydrocarbons are synthesised from fatty acids which have been mono- and di-unsaturated by acyl-CoA desaturases, chain lengthened by fatty acid elongases to give VLCFAs, and then subjected to oxidative decarbonylation to generate hydrocarbons. Recently, it was shown that the aldehyde oxidative decarbonylation step is catalyzed by the insect-specific cytochrome P450 enzyme *Cyp4g1* and its redox partner *Cpr*, both of which are strongly expressed in adult oenocytes (Qiu et al., 2012). Oenocyte-specific *Cyp4g1* knockdown results in the loss of most cuticular hydrocarbons, which leads to reduced adult viability and desiccation (Qiu et al., 2012).

Drosophila melanogaster shows sexually dimorphic cuticular hydrocarbons, with monoenes produced in males and dienes produced in females. *Desat1* catalyses the conversion of C16:0/C18:0 fatty acids into C16:1/C18:1, is expressed in both males and females, and is required for the synthesis of 7-unsaturated fatty acids (Dallerac et al., 2000; Wicker-Thomas et al., 1997). A second desaturase, *desatF,* is expressed specifically in female oenocytes and introduces a second double bond in the fatty acid precursors

of pheromones (Chertemps et al., 2006). The sex specificity of oenocytes in pheromone synthesis was illustrated by feminizing the oenocytes of adult male flies by overexpressing *transformer*. This stimulated diene hydrocarbon synthesis and elicited homosexual courtship from other males (Ferveur et al., 1997).

Recent studies have shown that age, diet and insulin signalling can all influence the cuticular hydrocarbon signature, providing important chemical information about an individual's nutritional status and reproductive fitness (Fedina et al., 2012; Kuo et al., 2012a; Kuo et al., 2012b). Young adult flies and flies with increased insulin signalling both display an increase in the proportion of short chain fatty acids which appears to promote sexual attractiveness (Kuo et al., 2012a; Kuo et al., 2012b).

Despite much research on the role of hydrocarbons in pheromonal communication and desiccation resistance, the source of fatty acid precursors for the synthesis of these cuticular hydrocarbons by oenocytes is still unclear. It also remains to be tested whether oenocytes take up lipids from the haemolymph, synthesise them *de novo*, or utilise both mechanisms in order to prevent large fluctuations in hydrocarbon levels in the face of different dietary nutrients. Furthermore, it is not known whether adult oenocytes play other roles in lipid metabolism relevant to nutrition and energy homeostasis. Neither is it known whether oenocytes regulate or are regulated by other lipid handling tissues such as the fat body and gut.

1.7 Aims

This thesis describes 4 main aims towards the general goal of understanding how adult oenocytes regulate lipid metabolism in *Drosophila*:

1. To develop a chemically defined medium that will allow the addition or subtraction of specific nutrients, such as amino acids, from the *Drosophila* diet.

2. To determine whether adult oenocytes are nutritionally regulated and, if so, by which dietary components.

3. To investigate how adult oenocytes regulate lipid metabolism relevant to nutrition and energy homeostasis.

4. To investigate organ-to-organ interactions between adult oenocytes and other lipid handling tissues such as the fat body.

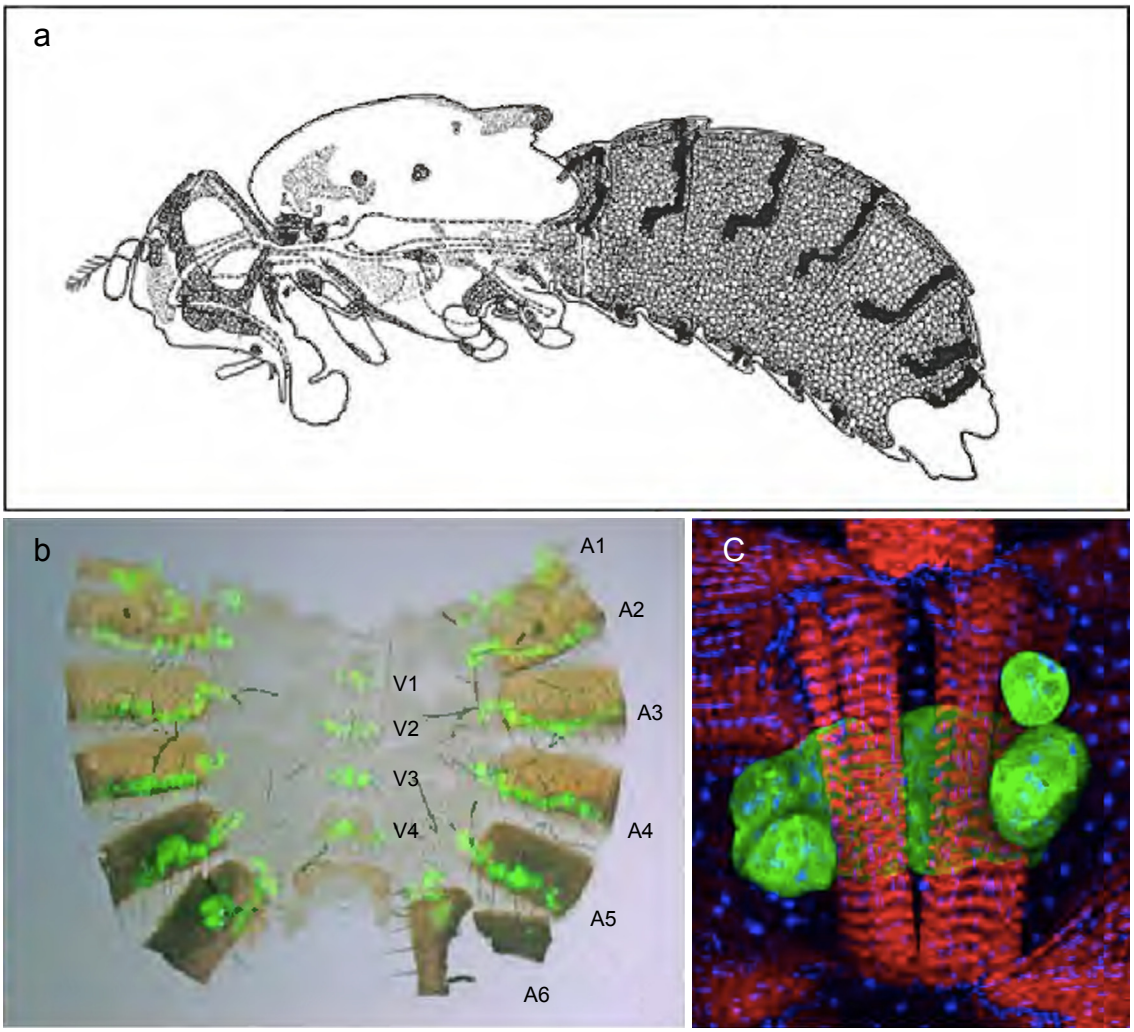
Figure 1.2 Adult oenocytes in *Drosophila melanogaster*

(a) Drawing of an adult fly showing the abdominal dorsal stripes and ventral clusters of oenocytes (black).

(b) Abdominal prep showing the segmental dorsal stripes and ventral clusters of oenocytes (green).

(c) Confocal image of ventral adult oenocyte cluster, V3 (green), associated with muscles (red) and nuclei (blue).

(Adapted from Makki *et al.*, In press)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CHAPTER TWO

Materials and Methods

CHAPTER TWO: Materials and methods

2.1 Drosophila stocks

The recombinant chromosomes *PromE(800)-GAL4,UAS-mCD8:GFP* (II) and *PromE(800)-GAL4,tubG80^{ts},UAS-mCD8:GFP* (II) (made by myself) were used for adult oenocyte specific manipulations. *PromeE(800)-GAL4* and *PromeE(800)-GAL4,tubG80^{ts}* were obtained from Joel Levine (Billeter et al., 2009). The following fat body drivers were used: *Lpp-GAL4,tubG80^{ts}* (S. Eaton, Max Planck Institue of Molecular Cell Biology and Genetics, Dresden, Germany), *Cg-GAL4,UAS-mCD8:GFP* (II) (made by myself), *Cg-GAL4* (Hennig et al., 2006). The following RNAi lines were used: *Cyp4g1RNAi^{KK105644}* (VDRC), *LpR1RNAi^{KK110259}* (VDRC), *Sam-SRNAi^{KK107709}* (VDRC), *Hnf4RNAi^{JF02539}* (TRiP). Other genetic elements used in this thesis include: *UAS-p60* (Weinkove et al., 1999), *UAS-Tsc1* and *UAS-Tsc2* (Tapon et al., 2001), *UAS-Rheb* (Powers et al., 2002), *UAS-Hid,UAS-Rpr* (I.M. Aliaga, MRC Clinical Sciences Centre, Imperial College London, London, UK), *UAS-InR^{K1409A}* and *UAS-HLH106$^{NTdel.3}$* (*UAS-SREBP^{DN}*) (Exelixis via Bloomington). A *w^{1118}* strain with isogenized I, II and III chromosomes (Iso 31), previously tested for normal development and behaviour, was used for CDD testing (Ryder et al., 2004).

2.2 Testing larval development on different CDDs

w^{1118} Iso31 larvae hatching within 2 hours were transferred either to the CDD diet or the control 2x media, at a density of 20 larvae/vial. Images of larvae 4 days after larval hatching (ALH) were taken at 1x magnification with the Leica DC 500 camera and Leica MZ16 F fluorescence microscope. Newly eclosed adult males were collected over a 12-hour time period and then weighed 24 hours later in groups of 5-20 using a CPA225D analytical balance.

2.3 Adult abdominal flat mount prep

Adult male flies were immobilised by immersing fly vials in ice for 1-2 minutes, and then tipped onto a cold glass plate resting on ice. Immobilised flies were dipped in 0.5% PBT and then transferred to a PBS-filled cell culture dish (Corning, Cat. No. 430165). Vannas spring scissors (Fine Science Tools, Cat. No. 15000-00) were used to dissect abdomens from the rest of the body, which were subsequently transferred using forceps to a drop of PBS on a silicon dish. Using the same scissors, a cut was made along the dorsal midline to open up the abdomens. The dissected abdominal cuticles were held open using insect pins placed in each of the four corners and then fixed in 4% formaldehyde/PBS for 1 hour. They were subsequently rinsed 3 times with PBS on the silicon dish and then transferred to glass wells containing PBS. Specimens were stained with DAPI (1:1000) and Alexa Fluor 555 Phalloidin (1:100) (Invitrogen, Cat. No. A34055) in 0.5% PBT for 1 hour followed by three 20 minute PBS washes. Specimens were stained with LipidTOX Deep Red neutral lipid stain (1:1000 in PBS) (Invitrogen, Cat. No. H34477) overnight at 4^{o}C. Abdominal cuticles were mounted in Vectashield (Vector Laboratories) and scored for LipidTOX-positive droplets.

2.4 EdU experiments

For 5-ethynyl-2′-deoxyuridine (EdU) experiments adult male flies were kept on 2ml of 2x food containing 0.05mM EdU, for 1 week continuously after eclosion. Vials were changed every 2-3 days. After 1 week abdominal cuticles were dissected and stained with DAPI (1:1000) in 0.5% PBT, followed by three 20 minute washes in PBS. Samples were then incubated for 1 hour with the Click-iT rx cocktail (200µl component F, 1720µl Rx buffer, 80µl $CuSO_4$, 5µl AlexaFluor 555), followed by three 20 minute washes in 0.5% PBT and a longer wash overnight in PBS. Abdominal cuticles were mounted in Vectashield (Vector Laboratories).

2.5 Microscopy and image analysis

All brightfield/fluorescence images, unless stated otherwise, were taken using an AxioCam HRc camera (Zeiss), Axiophot 2 microscope (Zeiss) and HBO 100 arc lamp. Lipofuscin images were taken using a ProgRes C14 camera (Jenoptik), Axioplan 2 microscope (Zeiss) and HBO 100 arc lamp. Confocal scans were taken using a Leica SP5 confocal microscope with a 1.5μm-step z-series. Photoshop CS5.1 (Adobe) and Fiji were used for image analysis.

2.6 Organ volume measurements

To calculate adult oenocyte volume confocal (Leica SP5) z stacks (1.5 μm spaced) were acquired of ventral oenocyte cluster V3. Volocity 5.5 (Improvision) software was subsequently used to analyse the images. The 'Find Objects' function was used to positively select for all GFP^+ oenocytes within the cluster, thereby providing total ventral cluster volume. To calculate the average oenocyte cell volume the total ventral cluster volume was divided by the number of cells within the cluster, calculated by manually counting the $DAPI^+$ nuclei of GFP^+ cells. Amira 5 (Visage Imaging) was used to calculate average fat body volume as described for average oenocyte volume. Confocal z stacks (1.5 μm spaced) were acquired of a readily identifiable fat body subset found just lateral to ventral oenocyte cluster V3 from a transgenic strain expessing GFP in the fat body (Cg>CD8::GFP). Total fat body subset volume was calculated by positively selecting for all GFP^+ cells throughout the stack. This value was then divided by the number of $DAPI^+$ nuclei within the subset to calculate the average fat body cell volume. For muscle and central brain total organ volumes were calculated. Phalloidin was used to stain the muscles overlying ventral oenocyte cluster V3, allowing positive selection and volume quantification using Amira. Adult central brains were stained using DAPI and then imaged using confocal microscopy (Leica SP5). Amira was used to analyse and quantify the 3D stacks by positively selecting for all $DAPI^+$ objects.

Diameters of Dilp2>mGFP-labeled mNSCs were calculated from the average of two orthogonal measurements using LAS AF software. Volumes were calculated using the formula: $V=\frac{4\pi r^3}{3}$

2.7 Lipid droplet quantification

Confocal microscopy was used to image a lateral subset of oenocytes from dorsal band A3, followed by lipid droplet quantification using Volocity 5.5 software (Improvision). This analysis was used to calculate total lipid droplet volume, expressed as a percentage of lateral oenocyte volume. The 8 most superficial z sections were initially selected and cropped from the 1.5μm confocal stacks to avoid changes in intensity due to tissue thickness. The 'find object', 'fill holes in object' and 'make ROI from object' functions were used together for the selection, and volume calculation, of GFP^+ oenocytes. The resulting ROI was cropped, thus allowing the volume calculation of lipid droplets present specifically within oenocytes. Lipid droplets were selected for volume analysis using the 'find object' function, together with an intensity threshold of above 60. Lipid droplet volume was divided by the total oenocyte volume and expressed as a percentage.

2.8 Gas chromatography-mass spectrometry analysis of TAG fatty acid methyl esters (FAMEs)

Adult male flies (in 500μl 0.9% saline) were homogenised by adding a ball bearing (Retsch) to each 2ml eppendorf tube and shaking for 3 min at 30/sec using a ball-mill homogenizer (Retsch). The homogenate together with the internal standard (TAG: Tripentadecanoin, 50μg/20μl. NEFA: Heptadecanoic acid, 20μg/20μl) were transferred to a screw thread tube (Fisher Scientific, FB59557), followed by 1.9ml 1:2 (v/v) chloroform:methanol, 0.625ml chloroform and 0.625ml dH_2O. Samples were centrifuged for 5 minutes at 1000rpm 14°C, followed by transfer of the bottom solvent phase to LP4 tubes using a glass pipette. For each sample a

second extraction was carried out by adding back authentic lower phase to the original screw thread tubes, followed by centrifugation for 5 minutes at 1000rpm. The bottom solvent phase from the second extraction was added to the initial solvent phase in the LP4 tube. The solvent phase was evaporated to dryness at 50°C under nitrogen (TurboVap LV evaporator from Zymark) before 1ml chloroform was then added to the dry lipid extract. Solid phase extraction (SPE) columns (Biotage) were pre-washed with 2 x 1ml acetone then with 2 x 1ml chloroform before samples were applied to the cartridge and allowed to drip through into collection tubes. A further 2 x 1ml chloroform was added to elute the CE/TAG fractions, followed by 2 x 1ml of diethyl ether with 2% acetic acid to elute the NEFA fraction. All fractions were dried down at 50°C under nitrogen. New cartridges were preconditioned with 4 x 1ml hexane before CE/TAG samples were dissolved in 1ml hexane. To elute the TAG fraction, the sample was first added to the cartridge and allowed to drip through, then washed with 2 x 1ml hexane:chloroform:ethyl acetate, before drying down at 50°C under nitrogen. 400µl toluene and 800µl 1.5% H_2SO_4 were added to all fractions, followed by 1 hour in a waterbath at 80°C, after which 2ml neutralising solution (0.125M $KHCO_3$ and 0.125M K_2CO_3) and 2ml cyclohexane were added. The samples were centrifuged at 2000rpm at 14°C before the solvent phase was transferred into LP4 glass tubes and dried down at 50°C under nitrogen. Samples were dissolved in 200µl chloroform and then transferred to GC vials. Samples were analysed using gas chromatography-mass spectrometry (Agilent 7890A GC, 5975C MSD, HP-INNOWax column). Column temperature method: Initial: Value 50°C, hold time 1 min. Ramp 1: Rate 20°C/min, value 190°C, hold time 6 min. Ramp 2: Rate 4°C/min, value 210°C, hold time 5 min. Ramp 3: Rate 10°C, value 240°C, hold time 2 min. GC/MSD ChemStation (Agilent) software was used for sample analysis. Peak integration was carried out using the ChemStation Integrator function. To calculate the total amount of lipid in each sample the following steps were carried out: 1. Integration values for all peaks (except for the internal standard) were summated. 2 The summation value was then divided by the internal standard peak integration value. 3 This value was

then multiplied by the mass of internal standard initially added to the sample. This value can be further divided by the mass of the sample to calculate the mass of lipid per mg fly.

2.9 Gas chromatography-mass spectrometry analysis of cuticular hydrocarbons

Individual male flies were immersed in 50μl of hexane, together with the internal standards (octadecane and hexacosane at 10ng/μl each), for 5 minutes. Glass pipettes were used to transfer the solvent to GC vials. Samples were analysed by GC-MS as described in section 2.8, using a DB-1+DG column. Column temperature method: Initial: Value 50°C, hold time 1 min. Ramp 1: Rate 10°C/min, value 150°C, hold time 0 min. Ramp 2: Rate 2°C/min, value 250°C, hold time 0 min. Ramp 3: Rate 10°C, value 325°C, hold time 10 min. Peak integration and software analysis was carried out as described in section 2.8

2.10 Statistical analysis

Student's t-test was used for statistical analyses involving two categories. One-way ANOVA together with Tukey's multiple comparison test was used for statistical analyses involving more than two categories.

CHAPTER THREE

Physiological markers of age in adult *Drosophila*

CHAPTER THREE: Physiological markers of age in adult *Drosophila*

3.1 New tools for studying physiology and ageing in adult *Drosophila*

Despite much research into the influence of nutrition on metabolism in *Drosophila*, an effective chemically defined diet allowing the precise manipulation of specific dietary components, has been missing. To address this gap, I set out to develop an amino-acid defined holidic diet.

Laboratory strains of *Drosophila* are kept on food containing autolysed yeast, glucose, cornmeal and agar, supplemented with live yeast sprinkles. These ingredients provide the full spectrum of nutrients required for the rapid development of *Drosophila* from an egg to a sexually mature adult in 10-12 days. We initially sought to optimise our control laboratory diet (1x yeast) using the rate of larval development as a sensitive readout. First, we omitted all live yeast sprinkles in order to allow the formulation of precise, uniform and reproducible yeast concentrations. Second, we doubled the autolysed yeast concentration (2x yeast), which resulted in a shorter developmental time of 4.5 days from larval hatching to white prepupa (WPP) and 9 days to adult eclosion. Hence, this 2x diet gives comparable developmental timing to the original recipe with live yeast sprinkles but is far more reproducible from vial-to-vial. All chemically defined diets (CDDs) subsequently synthesised were compared to this optimised 2x yeast diet. To test these CDD diets, I used a *Drosophila* strain isogenised for all three major chromosomes, w^{1118} Iso31, and selected for normal behaviour and lifespan, hereafter referred to as Iso31 (Ryder et al., 2004). My most successful formulation from more than 20 CDDs tested was based largely on previous defined recipes from Sang (Sang, 1956) and Rapport *et al.* (Rapport et al., 1983), using tryptone (a trypsin digest of casein) as the protein source (CDD 24). This yeast-free diet supported larval growth and development through to adult eclosion, albeit at a reduced rate compared to optimised 2x. Images taken after 4 days show that, despite significant growth, larvae on CDD 24 are somewhat smaller than those on 2x food (Fig. 3.1a,b). This smaller size is

coupled with a developmental delay of 2-2.5 days to reach both WPP and adult eclosion (Fig. 3.1e). Working towards the aim of formulating an amino acid-defined diet, I next replaced tryptone with individual amino acids (CDD 22). The ratios and amounts of amino acids were largely taken from Rapport *et al.* (Rapport et al., 1983) but I replaced 2'(3') nucleoside monophosphates with 5'(3') nucleoside monophosphates. This diet was also able to support larval growth and development through to adulthood but with a delay compared to CDD 24. Images taken after 4 days show significant larval growth as compared to *Drosophila* embryos (Fig. 3.1c,d). However, larvae on CDD 22 are smaller than those on both 2x yeast and CDD 24 (Fig. 3.1a-d). It takes 4.5-5 extra days to reach WPP compared to 4.5 days on 2x food (Fig. 3.1e). In summary, the number of days taken to reach WPP on CDD 24 is midway between those for 2x and CDD 22. It is important to note that on either CDD, all of the developmental delay occurs from larval hatching to WPP. Hence, the time taken from WPP to eclosion is constant at 4-4.5 days. On CDD 22, the larval developmental delay is associated with a significant 23.7% reduction (n=3, $p<0.01$) in the mass of eclosed adult flies, compared to flies raised on 2x yeast (Fig 3.1f). In conclusion, these results describe the successful formulation of an amino acid-defined diet that is able to support larval development through to adulthood, albeit with some delay.

3.2 Sucrose promotes larval growth more than fructose and glucose

Before utilising CDD 22 for dietary experiments, we sought to first optimise it by comparing the effect of three sugars on larval development and adult mass. To uncouple larval and adult specific effects, we initially raised all larvae on 2x yeast, transferred the newly eclosed adults to CDD 22 (containing either sucrose, fructose or glucose, all added at the same molar concentration), and then weighed the male adults 2 weeks later. Calculating mass per fly for each diet reveals no statistically significant difference between the three dietary sugars at the 5% significance level (Fig. 3.2a). Furthermore, considering the large differences in developmental timing between larvae raised on 2x or CDD 22, it is of great interest to note that

adults placed on these two diets for 2 weeks have very similar mass (7% reduction in mass on CDD 22, n=3-5, $p<0.01$, Fig. 3.2a). This result highlights that adult mass is predominantly determined by larval diet. In contrast, there is a significantly large difference between the mass of newly eclosed adults that were raised as larvae on 2x yeast or CDD 22 (1.6-1.8 fold higher mass on 2x yeast, n=3-5, $p<0.0001$), irrespective of the sugar used (Fig. 3.2b). Comparisons between the different dietary sugars reveal that there is a small but statistically significant increase (1.1 fold greater, n=3-5, $p<0.01$) in the mass of newly eclosed flies raised, as larvae, on CDD containing sucrose versus those on either fructose or glucose (Fig. 3.2b). This result indicates that, mole for mole, sucrose is the optimal of the three sugars for larval growth. Hence, sucrose was retained at the sole sugar component of CDD 22.

3.3 Amino acid drop-outs show that arginine is essential for larval growth

As a first step towards characterising the roles of individual amino acids on *Drosophila* physiology, I scored for lethality on a series of CDD 22 diets, each one lacking a different individual amino acid. For adult flies, consistent with previous studies, I observed that they are able to survive for a minimum of 4 weeks on a diet lacking all amino acids (CDD-aa) (data not shown). In contrast, scoring for larval lethality reveals which amino acids are essential for growth and development. After each of 18 amino acids had in turn been removed from the diet, they were scored as being either essential or non-essential for larval growth (Fig. 3.2c). Interestingly, the lists of essential and non-essential amino acids for *Drosophila* larvae is the same as for humans. The only notable exception is arginine, which is essential for larval growth but non-essential for adult humans. However, under conditions of rapid growth such as in preterm infants arginine is also essential in humans (Wu et al., 2004).

Figure 3.1 CDD 22 and CDD 24 support larval development

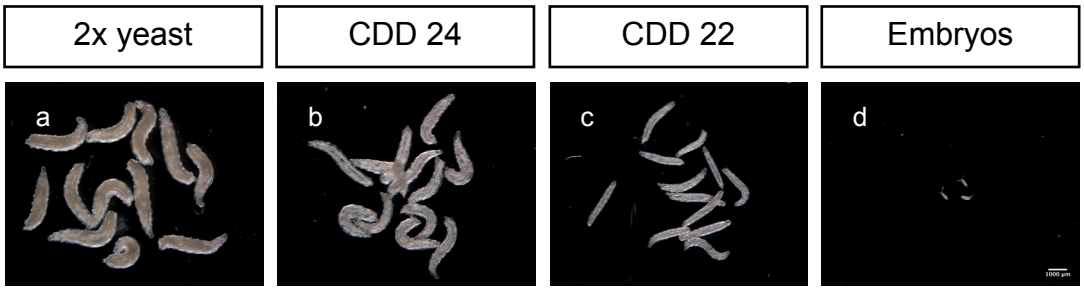
(a-c) Larvae 4 days after larval hatching (ALH) on (a) 2x yeast, (b) CDD 24 and (c) CDD 22. All images are taken at the same magnification.

(d) Embryos as a reference guide for larval growth.

(e) Days ALH to white prepupa (WPP) and eclosion on different diets.

(f) Mass of newly eclosed adult male flies kept as larvae on 2x yeast or CDD 22.

(** $p<0.01$)



e

Diet	Days ALH to WPP		Days ALH to eclosion	
	yw	Iso 31	yw	Iso 31
0.5X	8		14	
1X	6		12.5	
2X	5		11.5	
4X	5		11	
8X	4.5		10.5	
2X		4.5		9
CDD 24		6.5-7		11-11.5
CDD 22		9-9.5		13-13.5

f

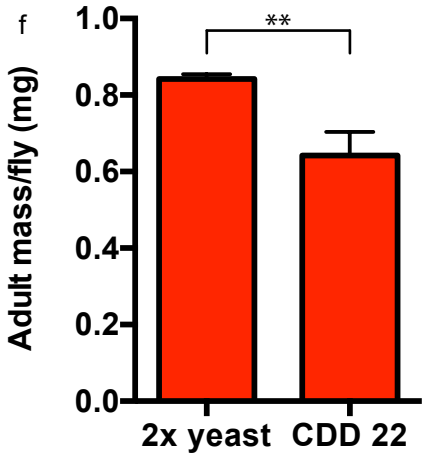


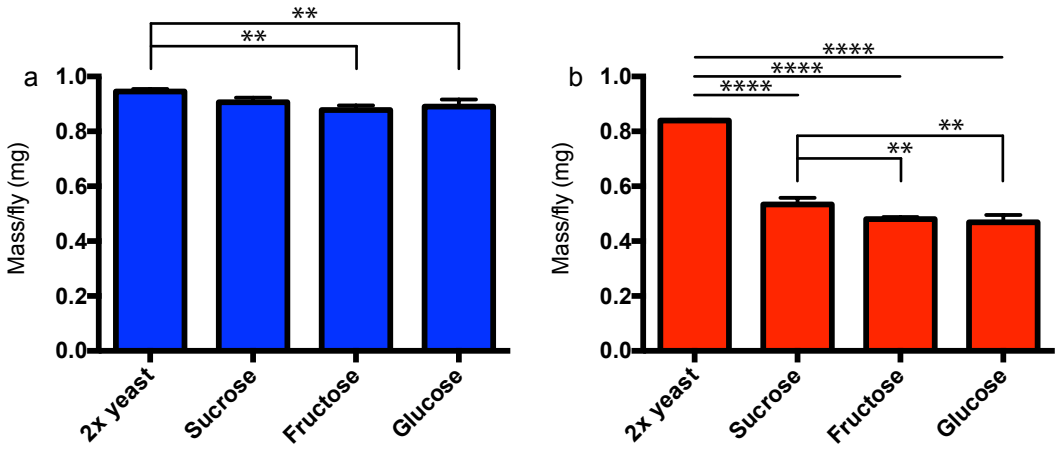
Figure 3.2 Sucrose is the optimal sugar for larval development

(a) Mass of 2-week-old adult male flies kept on CDD 22 containing either sucrose, fructose or glucose, at equimolar concentrations. Larvae were raised on 2x yeast.

(b) Mass of newly eclosed adult male flies raised as larvae on CDD 22 containing either sucrose, fructose or glucose, at equimolar concentrations.

(c) Essential vs non-essential amino acids for larval growth.

(** $p<0.01$, **** $p<0.0001$)



c

Essential vs non-essential amino acids for larval growth	
Essential	**Non-essential**
Arginine	Alanine
Histidine	Aspartic acid
Isoleucine	Cysteine
Leucine	Glutamic acid
Lysine	Glycine
Methionine	Proline
Phenylalanine	Tyrosine
Threonine	Serine
Tryptophan	
Valine	

3.4 Brown 'liver spots' and autofluorescence increase with age in oenocytes

Efforts to uncover the molecular mechanisms regulating lifespan require ageing-associated physiological markers. Several of these have already been described in adult *Drosophila*, including increased nucleolar fragmentation, lipofuscin accumulation, AGE accumulation and build up of muscle protein aggregates (Demontis and Perrimon, 2010; Larson et al., 2012; Luevano-Contreras and Chapman-Novakofski, 2010; Seehafer and Pearce, 2006; Singh et al., 2001; Terman and Brunk, 2004). Despite this, the links between global regulation of lifespan such as Insulin signalling and age-related markers in individual organs have not been well characterised (Partridge et al., 2011; Tatar, 2011). To address this deficit, I looked for age-related changes in individual organs as well as in the whole body.

First, age-related changes in mass were examined by comparing the weights of young (1 week old) and old (4 weeks old) adult male flies kept on the 2x diet. No statistically significant difference was observed between these two ages (n=4, p=0.364, data not shown). This demonstrates that adult mass does not change significantly with age. However, this is in contrast to a previous study that has shown that mass decreases with age (Bross et al., 2005). This discrepancy might be explained by different adult diets and/or by the longer time frame over which flies were weighed in the previous study (75 days versus 28 days). This raises the possibility that adult male flies could begin to lose mass, but, if so, this would be after 4 weeks of age.

To analyse adult lipid metabolising tissues, I developed an abdominal flat mount prep which involves the dissection of the abdominal cuticle with the fat body and oenocytes still attached. Figure 3.3a-d shows GFP-marked ventral oenocyte clusters from 0-4 week old adult male flies. Interestingly, light microscopy reveals that adult oenocytes become progressively darker with age (Fig. 3.3a'-d'). This oenocyte darkening results from a brown pigment within the oenocytes, which is undetectable at 0 weeks (eclosion) and first becomes faintly visible at 1 week of age (Fig. 3.3a',b'). Oenocyte pigment intensity increases with age so that, by 3-4 weeks, the oenocytes

are very darkly pigmented (Fig. 3.3c',d'). With this increase in brown pigmentation, I observed a concomitant age-dependent rise in oenocyte autofluorescence intensity under the red filter of the fluorescence microscope (Fig. 3.3a''-d''). This 'autofluorescence' is present in all oenocytes but not surrounding epidermal or fat body cells. Interestingly, the presence of brown pigmentation and autofluorescence is characteristic of lipofuscin, an ill-defined by-product of the lysosomal degradation pathway that has been shown in vertebrates to accumulate in multiple organs including the liver with ageing (Jung et al., 2007; Seehafer and Pearce, 2006; Terman and Brunk, 2004). As such, these results suggest that adult oenocytes may accumulate lipofuscin with age, providing a useful biological marker of adult age in *Drosophila*. It will be interesting to test whether other *Drosophila* tissues also accumulate lipofuscin with age.

3.5 Lipid droplets decrease in the fat body but accumulate in oenocytes with age

To investigate how lipid metabolism changes with age, we examined intracellular lipid droplets in both adult oenocytes and fat body, using a neutral lipid stain (LipidTOX). To mark the oenocytes, these experiments used a non-isogenic, recombinant fly strain, containing both the *PromE-GAL4* (expressed in oenocytes) and *UAS-CD8::GFP* transgenes. In young oenocytes (0-1 weeks) we observe almost no lipid droplets (Fig. 3.4a,b). However, at later stages (3-4 weeks), oenocytes exhibit a greatly increased number of lipid droplets (Fig. 3.4c,d). In light of the central role that the fat body occupies in lipid metabolism, I also asked whether any age-dependent fat body changes accompany those observed in oenocytes. I found that, in contrast to oenocytes, there is a dramatic decrease in lipid droplets with age (Fig. 3.4e-h). Although this has not been quantified from confocal images, the decrease appears to be most marked in terms of lipid droplet size rather than number. This observation is consistent with previous reports that total-fly TAG levels decrease with ageing (Skorupa et al., 2008). Together, these results demonstrate that, with increasing age, fat body lipid droplets

decrease in size, whereas oenocyte lipid droplets increase. These results also raise the interesting possibility that as the fly ages, more lipids are mobilised by the fat body and transported to the oenocytes for processing, resulting in a build-up of lipid droplets. This hypothesis will be developed further in chapter 6.

3.6 'Infected' oenocytes may not accumulate lipid droplets

I attempted to replicate the above results in a second strain, Iso 31, which is isogenised for chromosomes 1-3. Oenocytes from 4-week-old Iso 31 male flies exhibited brown pigmentation, as previously observed in *PromE-GAL4,UAS-CD8::GFP* flies (Fig. 3.5a). However, LipidTOX staining of Iso 31 flies revealed few if any oenocyte lipid droplets at 4 weeks of age (Fig. 3.5b). This demonstrates that two oenocyte markers of chronologically old flies, lipofuscin and lipid droplets, can be uncoupled by strain-specific differences. A clue as to why oenocyte lipid droplets may not accumulate with age in the Iso 31 strain is provided by the DAPI staining. DAPI positive objects, of non-uniform shape and uneven distribution, were observed within oenocytes of the Iso 31 strain (Fig 3.5b,b' arrows), but not the *PromEGAL4,UAS-CD8::GFP* strain (see Fig. 3.4). The morphology of these objects is highly reminiscent of intracellular protobacteria called Wolbachia that may regulate Insulin signalling in *Drosophila* (Ikeya et al., 2009; Iturbe-Ormaetxe and O'Neill, 2007). Indeed, PCR indicates that Iso 31 is infected with Wolbachia (I. Stefana and A. Gould, unpublished data). Thus, the possibility is raised that bacteria residing within the oenocytes of certain strains can influence age-dependent lipid metabolism.

Figure 3.3 Adult oenocytes accumulate lipofuscin with age

Adult oenocytes (*PromE-GAL4,UAS-CD8::GFP*, V2-4) from adult male flies 0-4 weeks after eclosion.

(a-d) Oenocytes (green). Stars indicate larval fat body (a).

(a'-d') Brown pigmentation within oenocytes increases with age.

(a''-d'') Oenocyte autofluorescence increases with age.

0 weeks
1 week
3 weeks
4 weeks
Oenocytes
Brown pigment
Autofluorescence
a
V2
V3
V4
b
c
d
a'
b'
c'
d'
a''
b''
c''
d''

Figure 3.4 Lipid droplets decrease in the fat body but accumulate in oenocytes with age

0-4 week old abdominal cuticles stained with LipidTOX (red).

(a-d) Adult oenocytes (*PromE-GAL4,UAS-CD8::GFP)* accumulate lipid droplets with age. Larval fat body cells are present in newly eclosed flies (a). All images (a-d) are taken at the same magnification.

(e-h) Lipid droplets decrease in the subcuticular fat body. All images (e-h) are taken at the same magnification.

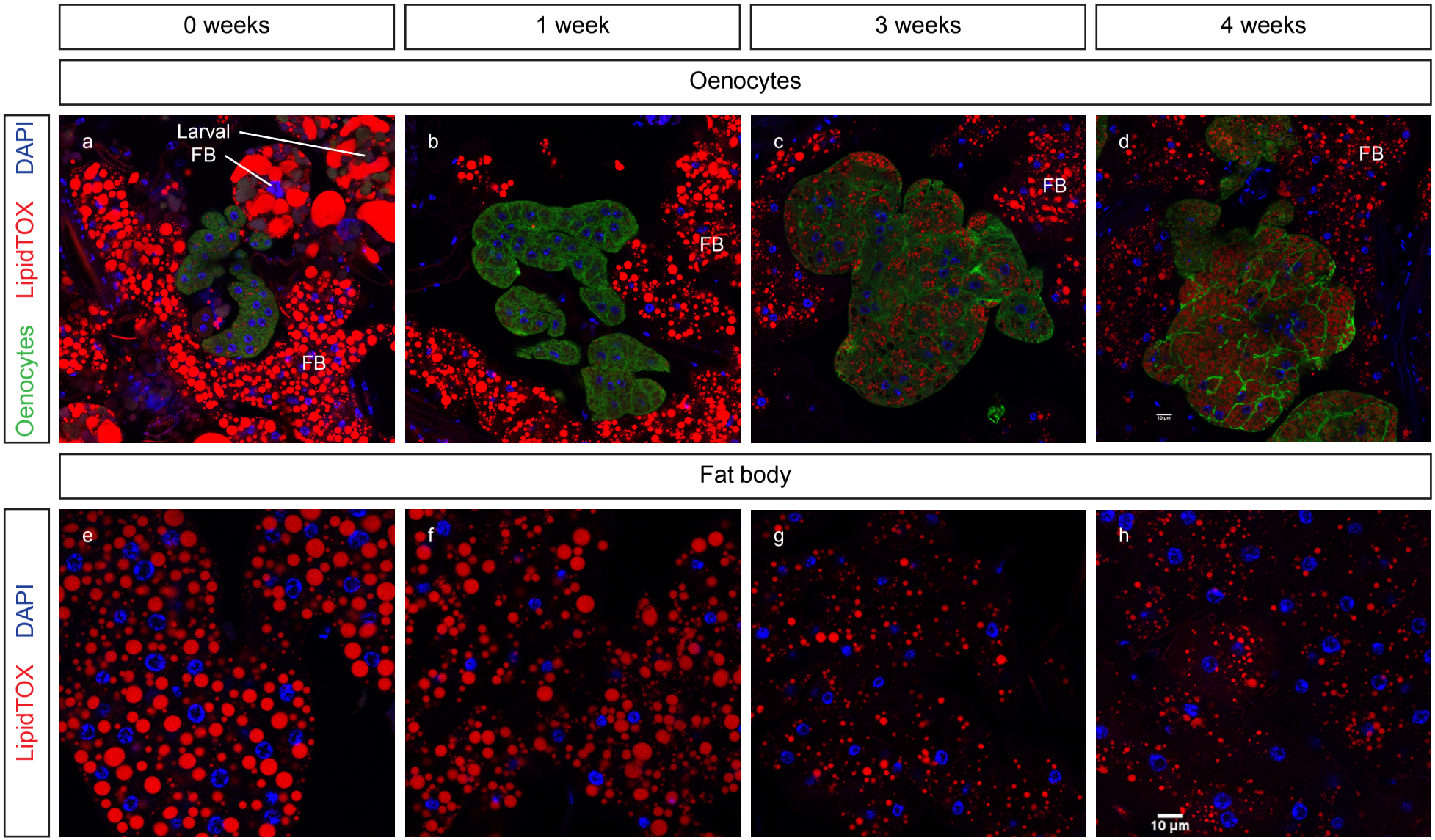
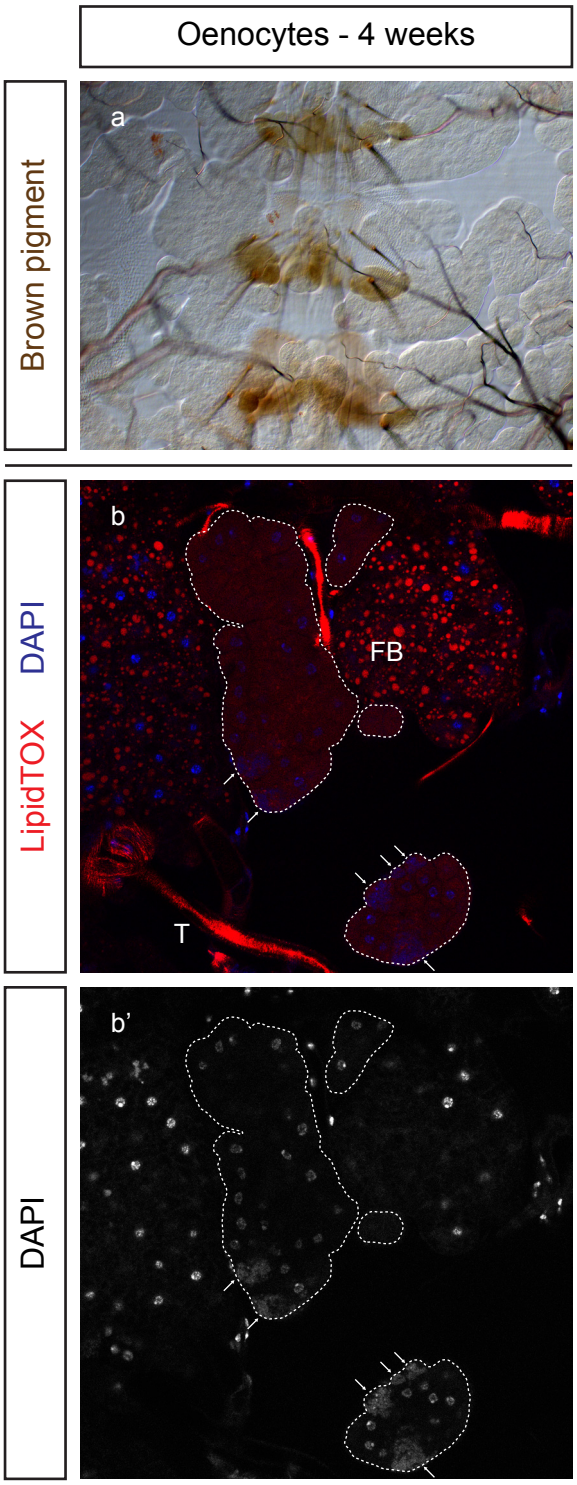

0 weeks
1 week
3 weeks
4 weeks
Oenocytes
Oenocytes LipidTOX DAPI
a
Larval
FB
FB
b
FB
c
FB
d
FB
Fat body
LipidTOX DAPI
e
f
g
h
10 µm

Figure 3.5 'Infected' oenocytes may not accumulate lipid droplets

(a) 4-week-old Iso 31 oenocytes (V2-4) contain age-dependent brown pigment.

(b) Iso 31 oenocytes (dotted lines) contain $DAPI^{+}$ objects (arrows) that may be Wolbachia. No $lipidTOX^{+}$ lipid droplets (red) are present.

T – Tracheole, FB – Fat Body


Oenocytes - 4 weeks
Brown pigment
a
LipidTOX DAPI
b
FB
T
DAPI
b'

3.7 Selective nutrient-dependent growth of oenocytes and fat body with age

Adult *Drosophila* body size is predominantly determined during the larval stages of life where most of the growth occurs. Even though body mass remains roughly constant during adulthood, it is not clear whether this masks growth at the level of individual organs. For example, a recent paper indicates that feeding during adult stages promotes growth of the midgut (O'Brien et al., 2011). To address this question, I screened a panel of different adult organs, in each case calculating volumes at 0 and at 2 weeks. From eclosion, male flies were maintained on either CDD 22 (CDD), or on CDD 22 lacking all amino acids (CDD-aa). No change from 0-2 weeks was observed in ventral abdominal muscle and central brain volume on CDD 22 (Fig. 3.6a). However, flies maintained on CDD-aa showed a statistically significant reduction in both central brain and muscle volumes when compared to volume at eclosion, 20% reduction (n=4-6, $p<0.01$) and 48% reduction (n=3, $p<0.05$) respectively (Fig. 3.6a). These results indicate that, although central brain and muscles do not grow during adulthood, they require dietary amino acids to maintain their size. The Insulin/IGF secreting cells of the CNS, the median neurosecretory cells (mNSCs), exhibit a 1.8 fold increase in soma volume (n=4-6, $p<0.0001$) during the first 2 weeks on CDD 22 (Fig. 3.6a). Removal of amino acids, however, leads to a 65% decrease (n=4-6, $p<0.001$) in original volume at eclosion (Fig. 3.6a). Therefore, in contrast to the central brain and muscles, mNSCs undergo amino acid-dependent growth during adulthood and remain sensitive to amino acid withdrawal. Intriguingly, both the key organs of the adipose axis, oenocytes and fat body, exhibited a striking volume increase during the first two weeks after eclosion. A 3.7 fold increase (n=4, $p<0.0001$) in oenocyte volume was closely matched by a 3.4 fold increase (n=3-5, $p<0.0001$) in fat body volume (Fig. 3.6a). For both these organs, there was no statistically significant change in volume between 0 weeks and 2 weeks on CDD-aa (Fig. 3.6a). These results indicate that the fat body and oenocytes require amino acids

for growth during adulthood but, in their absence, can maintain their volumes for at least 2 weeks.

Based on these results, we are able to group organs into three categories: 1. Those that do not grow during adulthood but require amino acids to maintain volume (e.g. central brain and muscles). 2. Those that require amino acids to grow during adulthood and also to maintain volume (e.g. mNSCs). 3. Those that grow during adulthood in an amino acid dependent manner but can maintain initial volumes without dietary amino acids (e.g. oenocytes and fat body). It will be interesting to determine whether fat body and oenocyte growth occurs only during the first 2 weeks, or whether it is sustained throughout adulthood. It is possible that the relatively small volumes at eclosion reflect an immaturity of function, as the larval fat body is still present for the first week of adulthood and so potentially capable of performing lipid and amino acid metabolic roles (Aguila et al., 2013; Aguila et al., 2007). In addition, gradual histolysis of the larval fat body might then promote lipid, carbohydrate and amino acid release which would reduce dietary requirements for these macronutrients. This could be tested by asking whether adult oenocytes and fat body still grow to the same extent upon inhibition of larval fat body histolysis.

3.8 Oenocyte growth responds differentially to dietary methionine drop-out

To further dissect the nutritional requirements of oenocytes for growth, I calculated organ volumes at 0 and 2 weeks, on diets lacking individual amino acids. Each of the 10 essential amino acids were, in turn, removed from CDD 22, as were 2 non-essential amino acids, glutamic acid and glycine. At 2 weeks of age, I observed no statistically significant difference in oenocyte volume between the complete CDD 22 and CDD 22-glutamic acid or CDD 22-glycine (Fig. 3.6b), demonstrating that growth was unaffected in the absence of these two non-essential amino acids. In contrast, oenocytes failed to grow on any of the diets lacking a single one of the essential amino acids, with no statistically significant difference between volume at eclosion

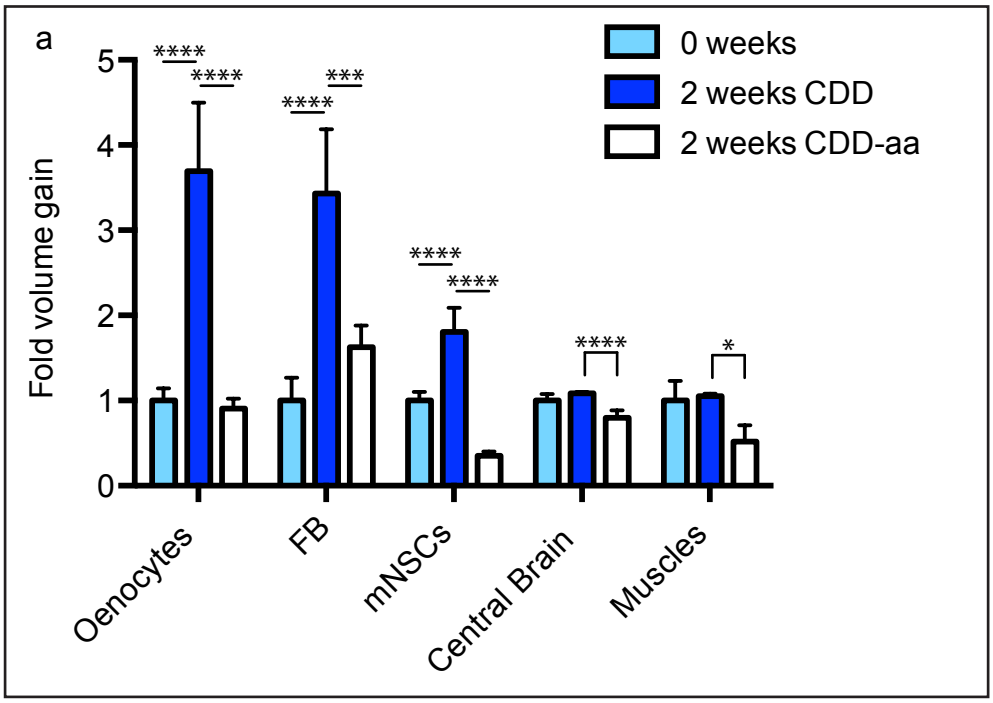
and at 2 weeks (Fig. 3.6b). Surprisingly, oenocytes were able to grow 2.7 fold (n=4-8, $p<0.001$) after eclosion on CDD-Met, to give a final average volume only 26% less than that on the complete CDD, which was not even statistically significant ($p>0.05$) (Fig. 3.6b). These results indicate that all the essential amino acids, except methionine, are strongly required in the diet for oenocyte growth. The unexpected effects of dietary methionine upon oenocytes are explored further in chapter 4.

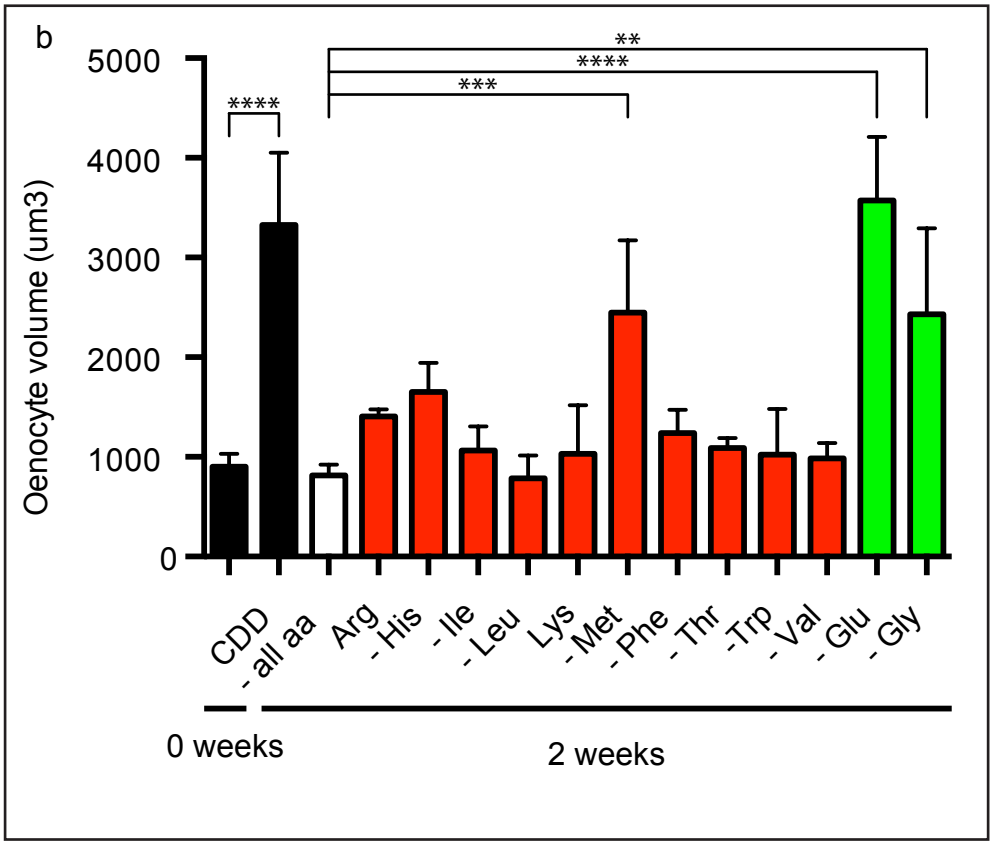
Figure 3.6 Selective nutrient dependent growth of oenocytes and fat body with age

(a) Organ volumes at eclosion and after 2 weeks on either CDD 22 or CDD 22-aa.

(b) Oenocyte volumes on CDD 22 lacking individual amino acids. Each of the 10 essential amino acids (red) were removed, as were 2 non-essential amino acids, glutamic acid and glycine (green).

(* $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$)


a
0 weeks
2 weeks CDD
2 weeks CDD-aa
Fold volume gain
0
1
2
3
4
5
****
****
****
***
****
****
****
*
Oenocytes
FB
mNSCs
Central Brain
Muscles


b
Oenocyte volume (um3)
0
1000
2000
3000
4000
5000
****
**
****
***
CDD
- all aa
Arg
- His
- Ile
- Leu
Lys
- Met
- Phe
- Thr
- Trp
- Val
- Glu
- Gly
0 weeks
2 weeks

3.9 Oral administration of EdU labels cytoplasmic puncta specifically within oenocytes

In polyploid tissues cell growth is usually accompanied by cycles of endoreplication. This lead me to ask whether adult oenocytes also undergo endoreplication during the first 2 weeks after eclosion, when they increase 3.7 fold in volume. To address this, adult flies were fed on a 2x yeast diet spiked with EdU (a thymidine analogue) for 1 week, after which they were dissected and mounted. Figure 3.7a shows multiple EdU positive nuclei in the posterior midgut and malphigian tubules, mounted as positive controls. Surprisingly, we also observed that a small proportion of fat body nuclei (fat body marked with GFP) had incorporated EdU into their DNA (Fig. 3.7b,c). Oenocyte nuclei did not incorporate EdU, indicating that genome-wide endoreplication does not occur (Fig. 3.7d,e). Intriguingly however, we observed numerous small EdU positive cytoplasmic puncta within the oenocytes (Fig. 3.7d,e). A much shorter EdU pulse (24hr at days 6-7) is still sufficient to observe the cytoplasmic Edu positive puncta (Fig. 3.7d-f), whose unknown identity is currently being investigated. Whatever the identity of the cytoplasmic puncta, I conclude that oenocytes do not replicate yet they increase in volume during the first 2 weeks of adulthood.

3.10 Oenocytes require TOR/PI3K signalling for growth without endoreplication

To explore the molecular basis of an increase in oenocyte volume without endoreplication, I tested the requirement for TOR/PI3K signalling. *UAS* responder lines for several TOR/PI3K components were crossed to the oenocyte driver *PromE-GAL4* (Billeter et al., 2009). To provide temporal specificity and to remove larval metabolism from the equation, a *PromE-GAL4,tubG80^{ts}* recombinant was constructed. At 18^{o}C this suppresses GAL4 activity but at 25-29^{o}C it is permissible for UAS gene expression. Furthermore, to measure organ volumes, a triple recombinant with *UAS-CD8::GFP* was generated.. Larvae were raised at 18^{o}C on 2x food, followed by

a temperature shift to 25°C at eclosion. Adults were kept at 25°C on CDD 22 for 2 weeks prior to dissection and oenocyte volume quantification.

Overexpressing a dominant-negative form of the Insulin Receptor (InR) reduced oenocyte volume to only 59% (n=3-4, $p<0.0001$) of controls (Fig. 3.8). Furthermore, blocking the PI3K pathway downstream of InR by overexpressing the PI3K subunit p60 resulted in a dramatic 77% reduction (n=4, $p<0.0001$) in final cell volume (Fig 3.8). These results demonstrate that oenocytes require InR and the PI3K signalling pathway for growth without endoreplication.

Blocking TOR activity by overexpressing Tsc1+2 (Tapon et al., 2001) resulted in a 53% reduction in oenocyte cell volume at 2 weeks, but this was not statistically significant at the 5% level (Fig. 3.9d). Intriguingly, LipidTOX staining of these oenocytes revealed ectopic lipid droplet accumulation, not observed in 2-week controls (Fig. 3.9a-b') nor when the Insulin/PI3K pathway was blocked (data not shown). Activating the TOR pathway by overexpressing Rheb in oenocytes led to a 1.8 fold cell volume increase (n=4, $p<0.01$) relative to controls (Fig. 3.9d,a,a',c,c'). Together, these results demonstrate that both the TOR and PI3K signalling pathways are required in a cell-autonomous manner for adult oenocyte growth without endoreplication. In addition, they suggest that TOR but not PI3K signalling is required to suppress inappropriate lipid droplet accumulation in oenocytes.

In conclusion, I have developed two useful tools for studying adult physiology. A chemically defined diet that supports development to adulthood and new physiological markers of age in the fat body and oenocytes. Using these, my results show that an age-dependent decrease in fat body stored lipids is accompanied by an increase in lipid droplets in oenocytes. They also demonstrate that oenocytes grow without nuclear genome duplication during adulthood, and that this occurs in an amino acid/TOR-PI3K dependent manner. The CDD and the physiological markers should also be useful for future studies of the influence of nutrition upon lifespan.

Figure 3.7 Adult oenocytes do not undergo endoreplication

Adult male flies were fed on 2x yeast spiked with EdU (red) for 1 week to examine whether oenocyte growth is correlated with genome-wide endoreplication.

(a) The posterior midgut (PMG) and malphigian tubules (MT), mounted as positive controls, contain multiple EdU^+ nuclei.

(b-c) A small population EdU^+ nuclei are present in the fat body (*Cg-GAL4,UAS-CD8::GFP*).

(d-f) Adult oenocytes do not contain EdU^+ nuclei. However, multiple EdU^+ cytoplasmic puncta are observed. These puncta are also present after just 24 hours of feeding on 2x yeast with EdU (f).

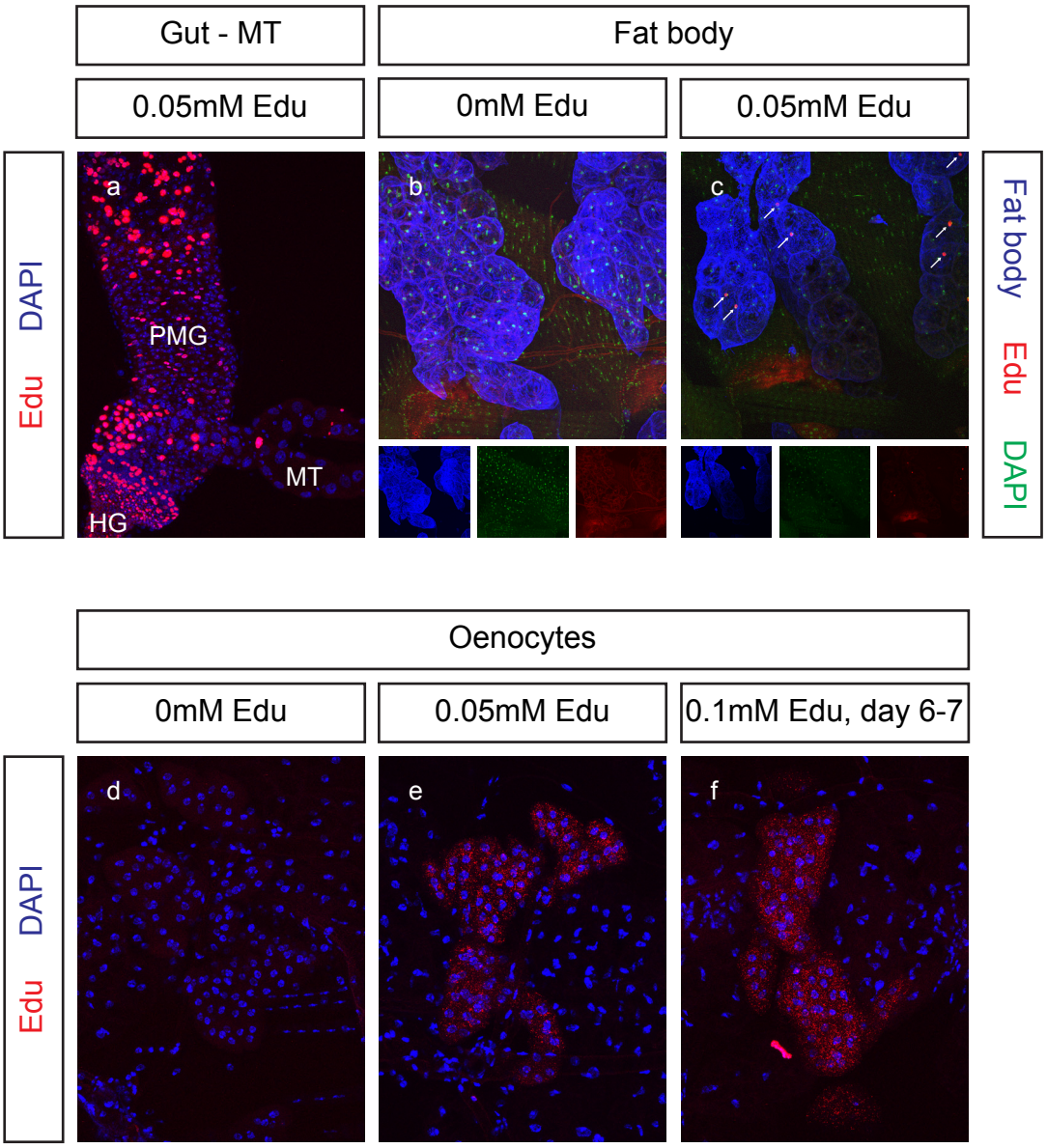

Gut - MT
Fat body
0.05mM Edu
0mM Edu
0.05mM Edu
Edu DAPI
a
PMG
MT
HG
b
c
Fat body Edu DAPI
Oenocytes
0mM Edu
0.05mM Edu
0.1mM Edu, day 6-7
Edu DAPI
d
e
f

Figure 3.8 Adult oenocytes require the InR/PI3K signalling pathway for growth

A requirement for the InR/PI3K signalling pathway in adult oenocyte growth was tested. Blocking this pathway (by overexpression of InR^{DN} or p60) results in reduced oenocyte volume in 2 week-old adult males.
(**** $p<0.0001$)

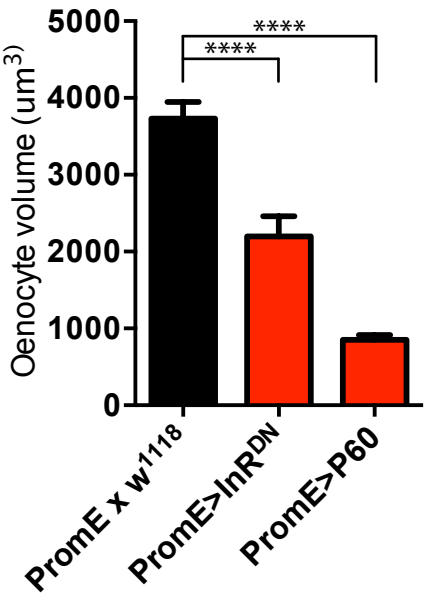
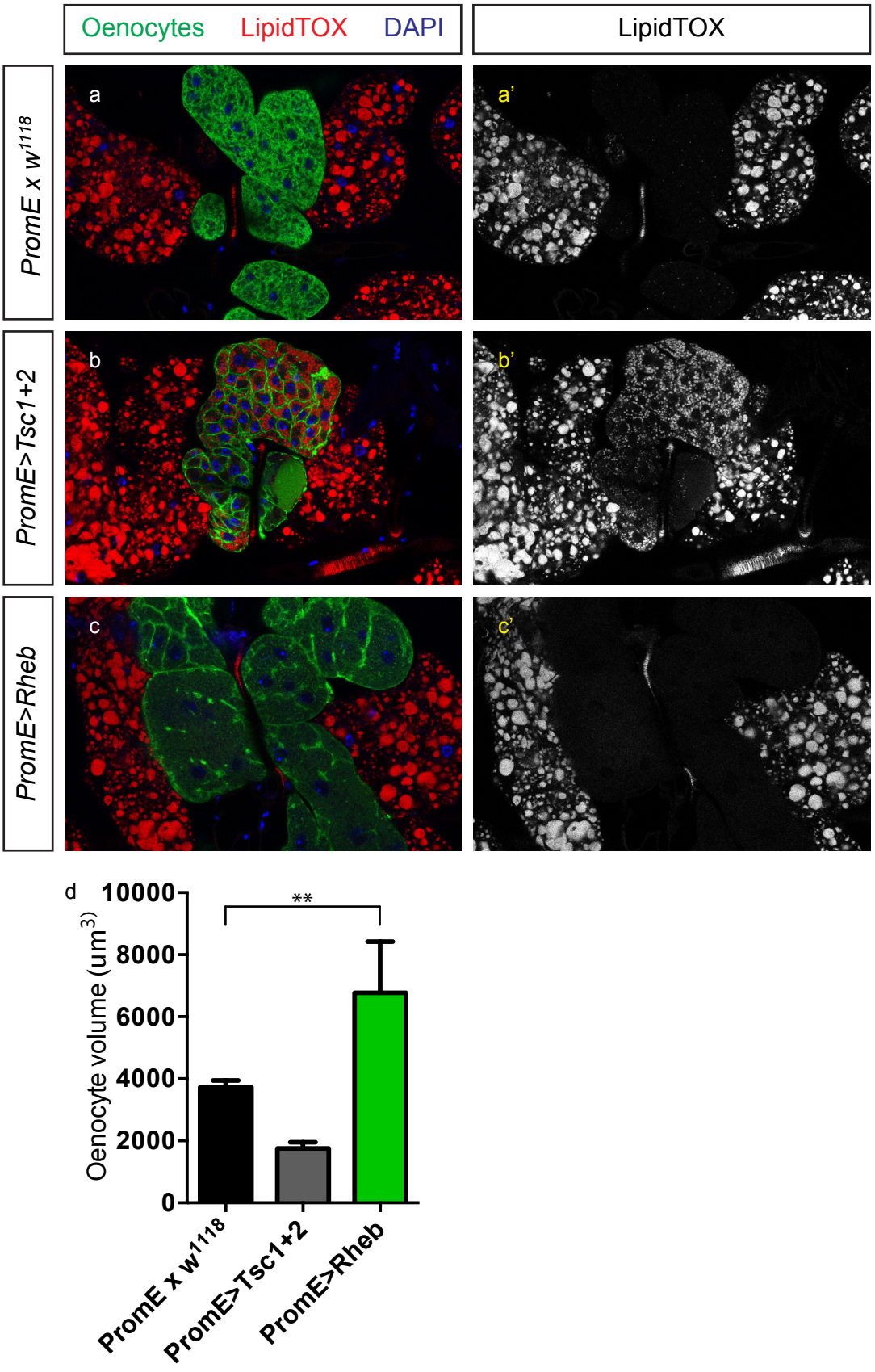

5000
4000
3000
2000
1000
0
Oenocyte volume (um3)
****
****
PromE x w1118
PromE>InRDN
PromE>P60

Figure 3.9 The TOR pathway is required for oenocyte growth and to suppress inappropriate lipid droplet accumulation

(a-c') Oenocytes (*PromE-GAL4,tubG80^{ts},UAS-CD8::GFP*) are stained with LipidTOX (red). Blocking the TOR pathway (*PromE>Tsc1+2)* results in lipid droplet accumulation (c-c').

(d) Adult oencoyte volume following suppression (*PromE>Tsc1+2*) and activation (*PromE>Rheb*) of the TOR pathway.

(** $p<0.01$)


Oenocytes
LipidTOX
DAPI
LipidTOX
PromE x w1118
PromE>Tsc1+2
PromE>Rheb
a
a'
b
b'
c
c'
d
Oenocyte volume (um3)
10000
8000
6000
4000
2000
0
**
PromE x w1118
PromE>Tsc1+2
PromE>Rheb

CHAPTER FOUR

Dietary methionine deficiency induces lipid overflow

CHAPTER FOUR: Dietary methionine deficiency induces lipid overflow

4.1 Adult flies raised on a methionine-deficient diet exhibit no significant changes in mass or global fatty acid levels

In mammals it has been shown that methionine-deficient diets promote hepatic steatosis, implicating methionine as a key regulator of lipid metabolism (Mato et al., 2008). Despite clear evidence in *Drosophila* that dietary methionine levels influence fecundity and lifespan (Grandison et al., 2009), it has been unclear whether there is a liver-like tissue in adult flies that accumulates lipids upon methionine deprivation. As a first step towards addressing this issue, I sought to examine global changes in adult fly lipids on methionine deficient diets. In this set of experiments, larvae were raised on a 2x yeast diet up to eclosion, after which they were transferred to either the complete CDD 22 (CDD), or CDD 22 minus methionine (CDD-Met). Flies were kept on these diets for 2 weeks before being weighed and processed for GC-MS analysis of TAG and non-esterified fatty acid (NEFA) content. No statistically significant difference in mass was observed between flies kept on the two diets (Fig. 4.1a). Furthermore, GC-MS analysis also revealed no statistically significant difference in total TAG content per mg (Fig. 4.1b). However, we observed a small (1.3 fold) but statistically significant (n=4, $p<0.05$) increase in total NEFA per mg (Fig. 4.1c). These results demonstrate that the global TAG content of adult flies is tightly buffered against changes in dietary methionine levels.

4.2 Dietary methionine but not choline deficiency induces ectopic oenocyte lipid droplets

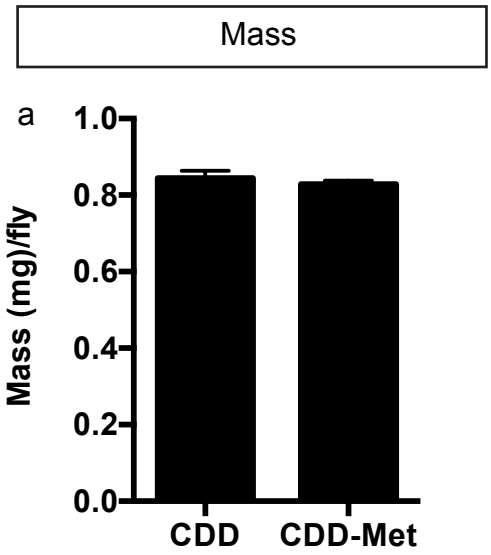
Despite no large changes in total TAG or NEFA content on CDD-Met, lipid droplet content and morphology were examined in oenocytes and fat body. Small, LipidTOX positive, lipid droplets were observed within the oenocytes of control flies at 2 weeks, comprising 6.7% of the total oenocyte volume (Fig. 4.2a,a',e). However, flies kept on CDD-Met showed a statistically

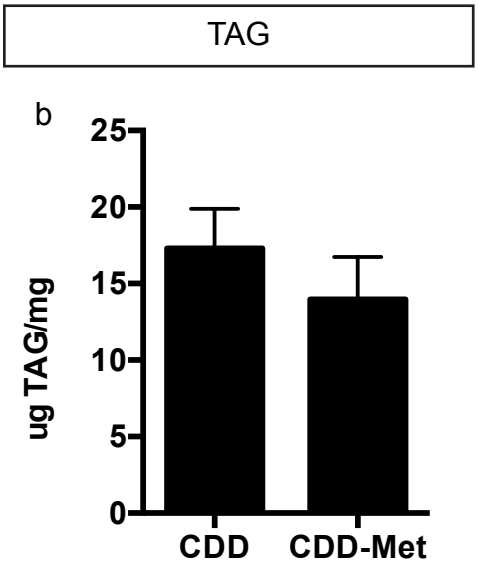
significant 2.6 fold increase in the percentage of oenocyte volume occupied by LDs (Fig. 4.2a,a',c,c',e). In addition, much larger lipid droplets were observed in the fat bodies of flies kept on CDD-Met (Fig. 4.2a,a',c,c'). As a next step, flies were kept on a diet lacking both methionine and choline (CDD-Met-Chol), commonly used in mammalian studies of hepatic steatosis (Basaranoglu et al., 2010; Koteish and Diehl, 2001). On this diet, as on CDD-Met, we observed much larger lipid droplets in the fat body (Fig. 4.2a,a',c-d'). The oenocytes also exhibit an even greater statistically significant increase ($p<0.001$) of 3.9 fold in the percentage lipid droplet: oenocyte volume as compared to the control CDD. Oenocytes on CDD-Met-Chol have a 1.5 fold increase in the percentage of lipid droplet: oenocyte volume when compared to CDD-Met alone, but this was not statistically significant at the 5% level (Fig. 4.2e). To address whether any of these effects can be mediated by choline alone, we kept flies on a diet lacking choline (CDD-Chol) for 2 weeks. On this diet, however, we failed to observe either the increase in fat body lipid droplet size or the oenocyte steatosis. These results show that most of the observed effects on LDs are mediated by methionine rather than choline deficiency (Fig. 4.2a-b',e).

Together, these results demonstrate that a lack of methionine is able to promote oenocyte steatosis. This suggests that adult oenocytes may share at least some aspects of metabolic regulation with the mammalian liver. It is intriguing that the increase in fat body lipid droplet size on CDD-Met does not correlate with an increase in whole body TAG content. The reason for this is not yet clear but it could reflect an increase in non-TAG components of LDs (e.g. cholesterol esters).

Figure 4.1 Mass and TAG content are tightly buffered against changes in dietary methionine levels

Adult male flies were kept on either CDD 22 or CDD 22-Met for 2 weeks. (a) There is no difference in mass (a) or TAG content (b) between flies kept on CDD 22 or CDD 22-Met. There is small increase in NEFA content (c).
(* $p<0.05$)


Mass
a
Mass (mg)/fly
1.0
0.8
0.6
0.4
0.2
0.0
CDD
CDD-Met


TAG
b
ug TAG/mg
25
20
15
10
5
0
CDD
CDD-Met

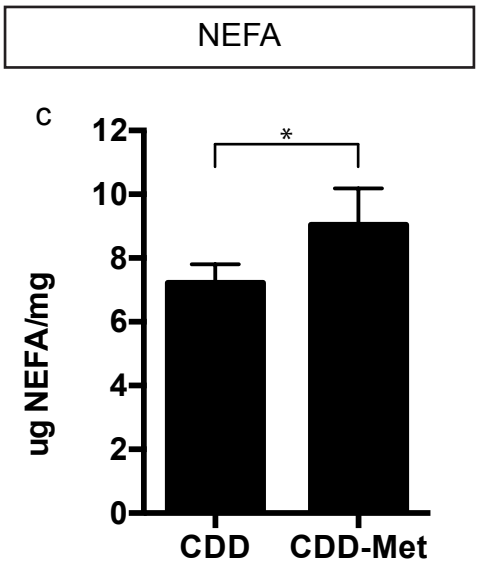

NEFA
c
ug NEFA/mg
12
10
8
6
4
2
0
*
CDD
CDD-Met

Figure 4.2 Dietary methionine deficiency results in lipid droplet accumulation within oenocytes

Oenocytes (*PromE-GAL4,tubG80^{ts},UAS-CD8::GFP*) and fat body are stained with LipidTOX (red).

(a) Adult oenocytes and fat body on the control CDD.

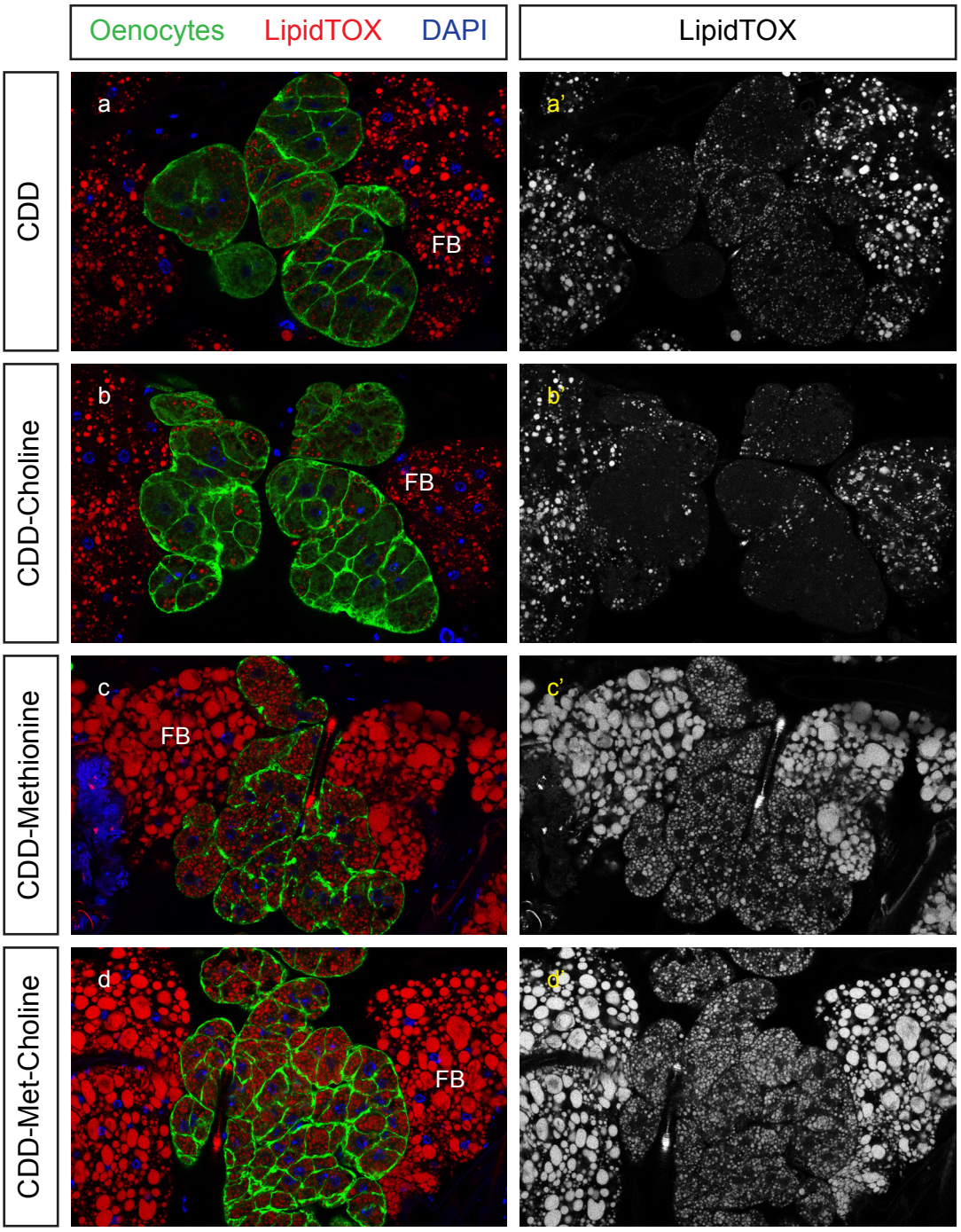
(b) Dietary choline deficiency has no effect on oenocyte and fat body lipid droplet number or morphology.

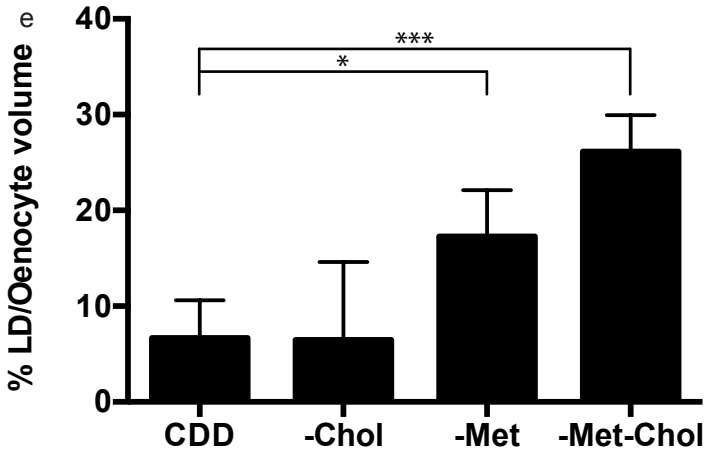
(c) Dietary methionine deficiency induces lipid droplet accumulation in oenocytes. Fat body lipid droplets also increase in size.

(d) CDD-Met-Choline has a similar phenotype to CDD-Met.

(e) Lipid droplet volume as a percentage of total oenocyte volume.

(* $p<0.05$, *** $p<0.001$)


Oenocytes
LipidTOX
DAPI
LipidTOX
CDD
CDD-Choline
CDD-Methionine
CDD-Met-Choline
a
a'
b
b'
c
c'
d
d'
FB


e
% LD/Oenocyte volume
40
30
20
10
0
***
*
CDD
-Chol
-Met
-Met-Chol

4.3 Methionine deficiency is exacerbated by defective one carbon metabolism

One carbon metabolism has recently been shown to be key regulator of SREBP-1-dependent lipogenesis (Mato et al., 2008). In this cyclical series of enzymatic reactions, methionine is first converted to S-adenosylmethionine (SAM), a universal methyl donor, before subsequent conversions to S-adenosylhomocysteine (SAH) and homocysteine. Homocysteine can be remethylated to generate methionine, or it can enter the transsulfuration pathway whereby cysteine, taurine and glutationine are synthesised. To investigate whether the effects of CDD-Met on adult oenocytes are signalled via this pathway, S-adenosylmethionine synthetase (*SAM-S*), the enzyme required for the conversion of methionine to SAM, was knocked down using RNAi. The same temperature shift and dietary regimens were adopted as described in section 3.10. Combining brightfield with fluorescence microscopy, we observed that oenocytes from adult flies kept on either CDD or CDD-Met, looked structurally very similar at the gross anatomical level (Fig. 4.3a-b'). In addition, experimental flies carrying the *UAS-SAM-S RNAi* transgene, on CDD, also showed no striking differences in oenocyte number or morphology compared to the controls (Fig. 4.3a,a',c,c'). However, experimental flies kept on CDD-Met displayed a striking partial ablation phenotype, with most of the GFP^+ oenocytes absent (Fig. 4.3d,d'). The absence of oenocytes was confirmed with brightfield observations. This shows a strong interaction between loss of SAM-S activity and dietary methionine deficiency. Combining both manipulations (*PromE>SAM-S RNAi* on CDD-Met) may block flux through the one carbon metabolism pathway resulting in oenocyte loss Further experiments would be required to ascertain whether oenocyte disappearance occurs via apoptosis or an alternative mechanism.

4.4 Lipophorin receptor 1 mediates oenocyte steatosis on a methionine deficient diet

Lipophorin receptor-mediated lipid uptake was examined as a possible mechanism contributing to oenocyte steatosis on CDD-Met. Lipid uptake in *Drosophila* is mediated by two lipophorin receptors, LpR1 and LpR2, which are both expressed in embryonic oenocytes (Gutierrez et al., 2007). To investigate the role of LpR1, *PromE-GAL4,tubG80^{ts},UAS-CD8::GFP* and *UAS-LpR1 RNAi* flies were crossed. Larvae from this cross were reared at 18^{o}C on 2x food until adult eclosion, after which they were transferred to CDD or CDD-Met and kept for 2 weeks at 29^{o}C. Knockdown of *LpR1* specifically within adult oenocytes results in an 85.5% reduction in total oenocyte lipid droplets on the control CDD (n=3-4, p<0.05) (Fig. 4.4a,a',c,c',e). Importantly, *LpR1* knockdown also blocks lipid droplet accumulation in oenocytes on CDD-Met, resulting in a 79.4% reduction in total lipids (n=4, p<0.0001) (Fig. 4.4b,b',d,d',e). These results demonstrate that LpR1 is required for oenocyte lipid droplet accumulation on both the control and experimental (CDD-Met) diets. This suggests that increased haemolymph lipids, taken up via LpR1, contribute to oenocyte steatosis on methionine deficient diets.

Figure 4.3 Inhibition of the one carbon metabolism cycle results in oenocyte loss

Ventral oenocytes (*PromE-GAL4,tubG80^{ts},UAS-CD8::GFP*, V1-V4). Trachae can be seen arising from the spiracles and extending towards the ventral midline.

(a-a') Adult oenocytes on the control CDD.

(b-c') No effect on gross oenocyte morphology is observed upon dietary methionine deficiency (b'b'), or inhibition of one carbon metabolism (*PromE>SAM-S RNAi)* on CDD (c-c').

(d-d') *PromE>SAM-S RNAi* on CDD-Met leads to a partial ablation phenotype.

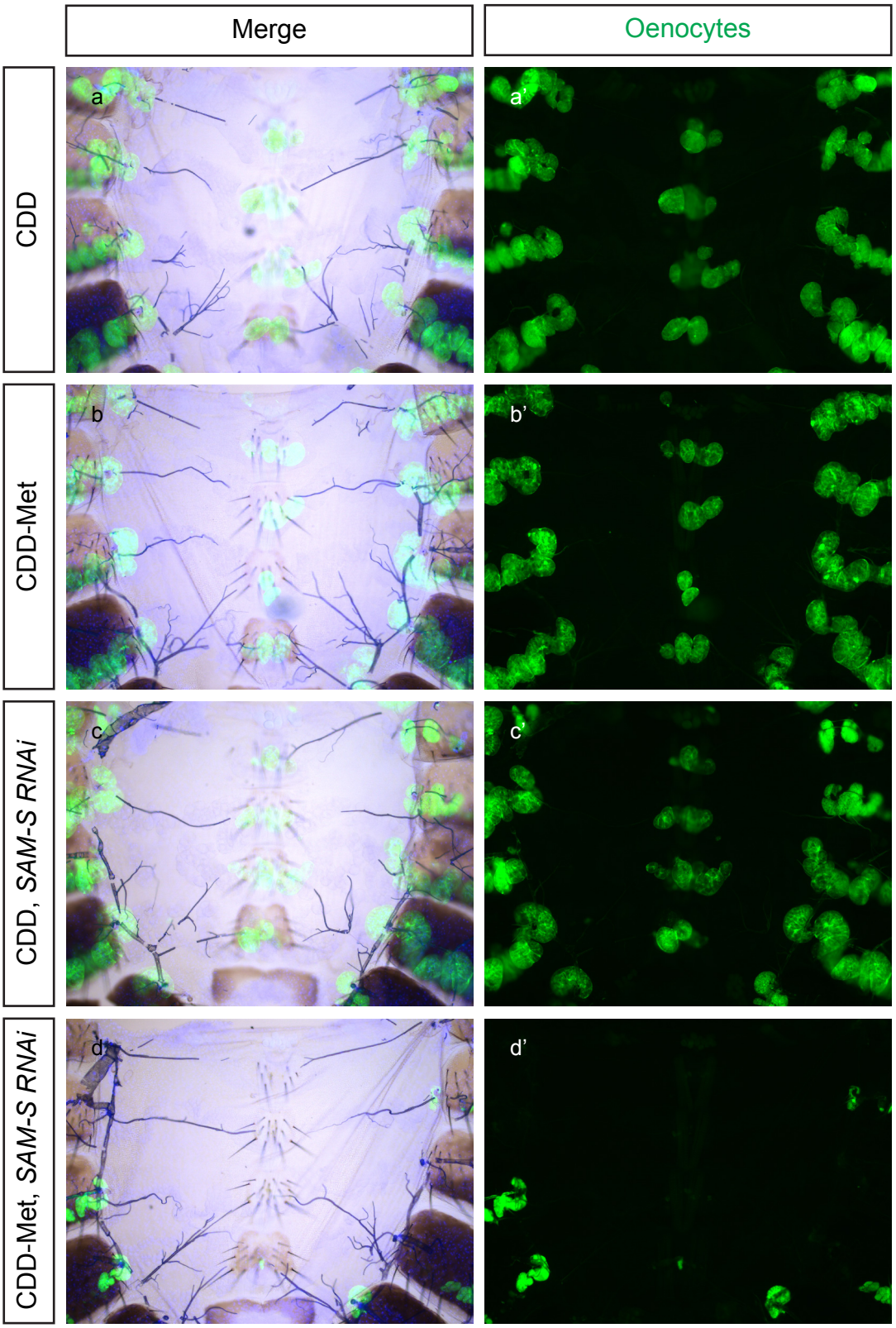
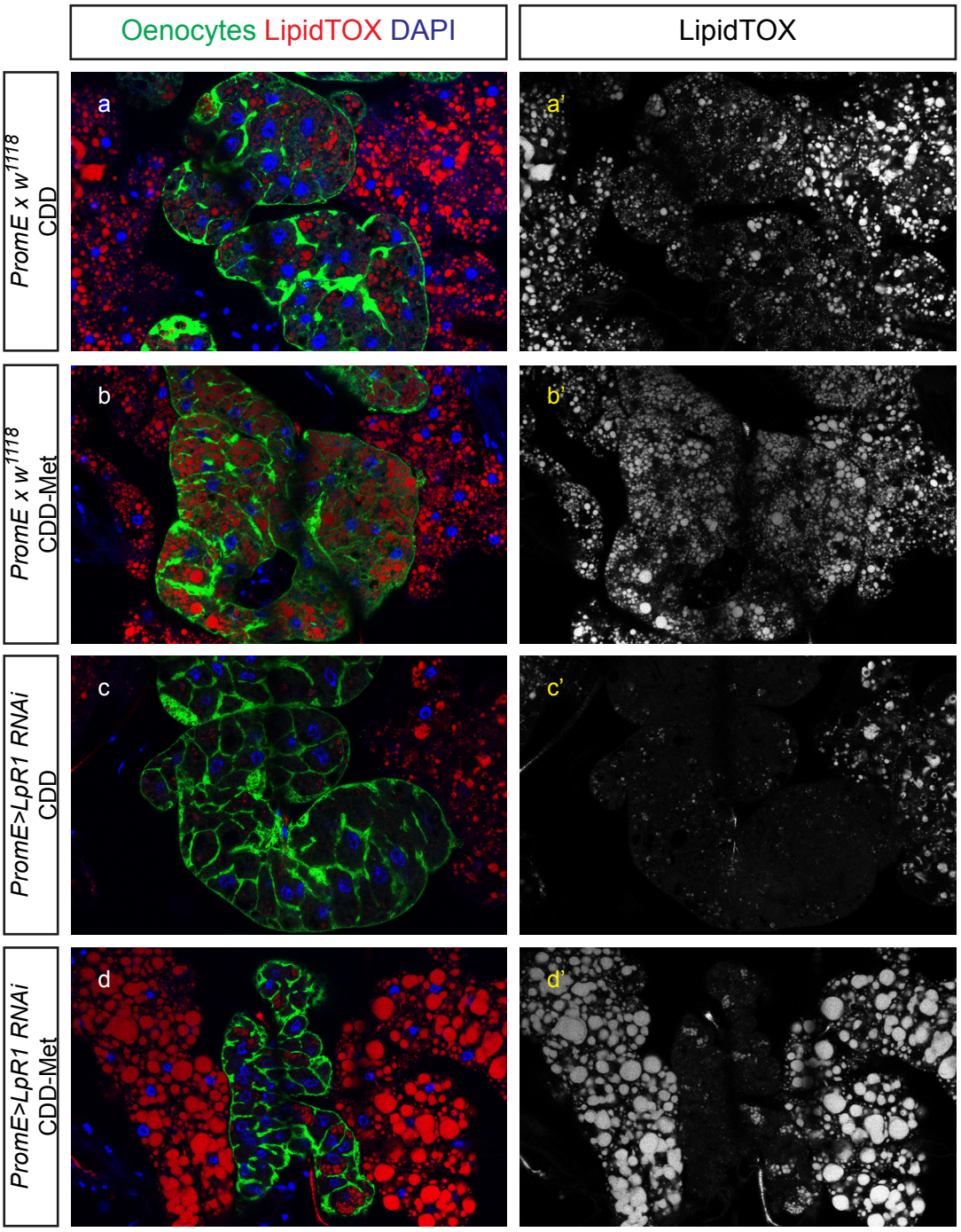
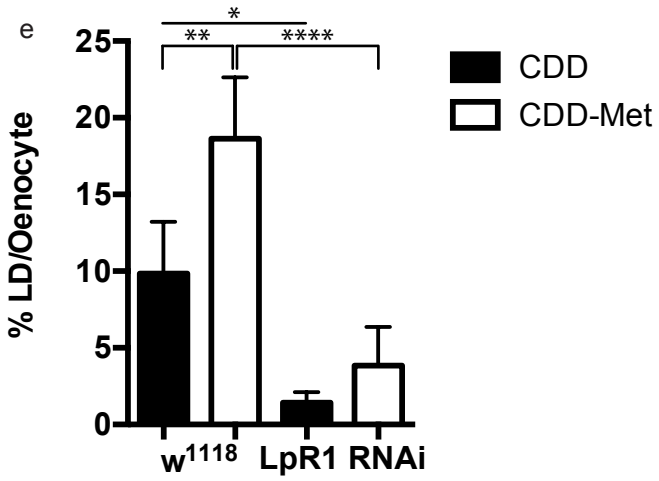

Merge
Oenocytes
CDD
CDD-Met
CDD, SAM-S RNAi
CDD-Met, SAM-S RNAi
a
a'
b
b'
c
c'
d
d'

Figure 4.4 LpR1 is required for lipid uptake on CDD and CDD-Met

Oenocytes (*PromE-GAL4,tubG80^{ts},UAS-CD8::GFP*) and fat body are stained with LipidTOX (red). LpR1knockdown (*PromE>LpR1 RNAi*) prevents lipid droplet accumulation on both CDD and CDD-Met (a-d').

(* $p<0.05$, ** $p<0.01$, **** $p<0.0001$)


Oenocytes LipidTOX DAPI
LipidTOX
PromE x w1118 CDD
PromE x w1118 CDD-Met
PromE>LpR1 RNAi CDD
PromE>LpR1 RNAi CDD-Met
a
a'
b
b'
c
c'
d
d'


e
*
**
****
25
20
15
10
5
0
% LD/Oenocyte
CDD
CDD-Met
w1118
LpR1 RNAi

CHAPTER FIVE

Functions of oenocytes revealed by genetic ablation

CHAPTER FIVE: Functions of oenocytes revealed by genetic ablation

5.1 Establishment of a genetic system to ablate adult oenocytes

Ablation is a classical approach, widely used to elucidate the function of specific organs or cell types. This technique was initially adapted in the lab, to investigate the function of larval oenocytes, making use of the GAL4/UAS system to overexpress *reaper*, a component of the *Drosophila* apoptotic pathway (Brand and Perrimon, 1993; Gutierrez et al., 2007). The GAL4 driver used, *BO-GAL4,* and several other drivers for larval oenocytes do not express specifically in adult oenocytes (Gutierrez et al., 2007). Perhaps this is not surprising as the larval and adult oenocytes have quite separate developmental origins (Gould et al., 2001). Fortunately however, the *PromE-GAL4* driver had already been developed to ablate adult oenocytes, revealing their role in cuticular hydrocarbon synthesis (Billeter et al., 2009). To achieve efficient ablation, *PromE-GAL4* was crossed to a recombinant *UAS-hid,UAS-rpr* responder line. To achieve precise temporal specificity, the GAL4 line was recombined with *tub-G80^{ts}*. Furthermore, I developed an optimal temperature shift regime. I raised animals at 18^{o}C until eclosion, followed by 29^{o}C for 2 days, then 25^{o}C thereafter. This ensures that maximal activity of both apoptotic genes becomes derepressed only during early adulthood, and allows subsequent adult development to occur at 25 ^{o}C, and not 29^{o}C. This is relevant as pilot studies indicated some differences in lipid metabolism between Iso 31 flies raised at 29^{o}C (data not shown).

Adult male flies at 6 days of age were dissected for control and experimental genotypes and abdominal preps analysed. GFP fluorescence from the control genotype (*PromE-GAL4,tub-G80^{ts},UAS-CD8::GFP*) reveals the characteristic dorsal oenocyte bands (A1-A6), together with the 4 four ventral oenocyte clusters (Fig. 5.1a,a'). In contrast, experimental adults (*PromE-GAL4,tub-G80^{ts},UAS-CD8::GFP;UAS-hid,UAS-rpr*) displayed a dramatic reduction in the number of GFP^{+} cells, indicating a highly efficient ablation of all oenocytes (Fig. 5.1b,b'). Nevertheless, some small GFP^{+} cells could still be discerned after 6 days in the experimental genotype. However, high

magnification views indicate that all of the residual oenocytes show characteristic signs of apoptosis, such as reduced nuclear size and small, irregularly shaped GFP^+ material (Fig 5.1c,d). Together, these results demonstrate that the *PromE-GAL4/UAS-hid,UAS,rpr* system can be used to eliminate efficiently most or all functional adult oenocytes.

5.2 Efficient oenocyte ablation leads to adult lethality after about 1 week

Billeter *et al.* were able to conduct behavioural studies on viable adults lacking a proportion of their oenocytes but retaining some (Billeter et al., 2009). We therefore sought to test whether more efficient oenocyte ablation would produce a stronger adult phenotype. I observed that adults carrying *PromE-GAL4,tub-G80^{ts},UAS-CD8::GFP* and *UAS-hid,UAS-rpr*, and subjected to the optimised temperature shift protocol were viable but began to die from day 7 onwards (data not shown). This strongly suggests that oenocytes play an essential role in the adult, although I cannot rule out lethality mediated by leaky *PromE-GAL4* expression in the ejaculatory bulb (Billeter et al., 2009).

5.3 The tracheal system of oenocyte-ablated flies is structurally intact at the gross anatomical level

Both larval and adult oenocytes are closely associated with fine tracheal branches or tracheoles. Moreover, it has been recently shown that larval oenocytes are required for tracheal function (Parvy et al., 2012). In their absence, larvae become hypoxic and this is therefore likely to influence metabolism. To ask whether adult oenocytes play a similar regulatory role with respect to the tracheal system, I used brightfield microscopy to examine tracheal branching in control and oenocyte-ablated 6-day-old adults. In wild type flies tracheal branches surrounded by fat body, extend from the spiracle of abdominal segments towards the ventral oenocyte clusters. However, no defects were observed in this tracheal branching

pattern or in tubular morphology in oenocyte ablated flies (Fig. 5.2a-c). This result strongly suggests that tracheal integrity is not dependent upon oenocytes in the same way in adults as it is in larvae. To confirm the lack of hypoxia in oenocyte ablated flies, whole-body lactate measurements and hypoxia reporters could be used.

Figure 5.1 Establishment of the GAL4/UAS-hid,rpr system for oenocyte ablation

(a-a') Adult cuticle without *UAS-hid,UAS-rpr* displays the normal number and pattern of adult oenocytes.

(b-b') *UAS-hid,UAS-rpr* produces efficient ablation of adult oenocytes.

(c) Magnification of V3 from the control genotype.

(d) High magnification view of V3 from oenocyte-ablated flies shows characteristic signs of apoptosis, such as reduced nuclear size and small, irregularly shaped GFP^+ material.

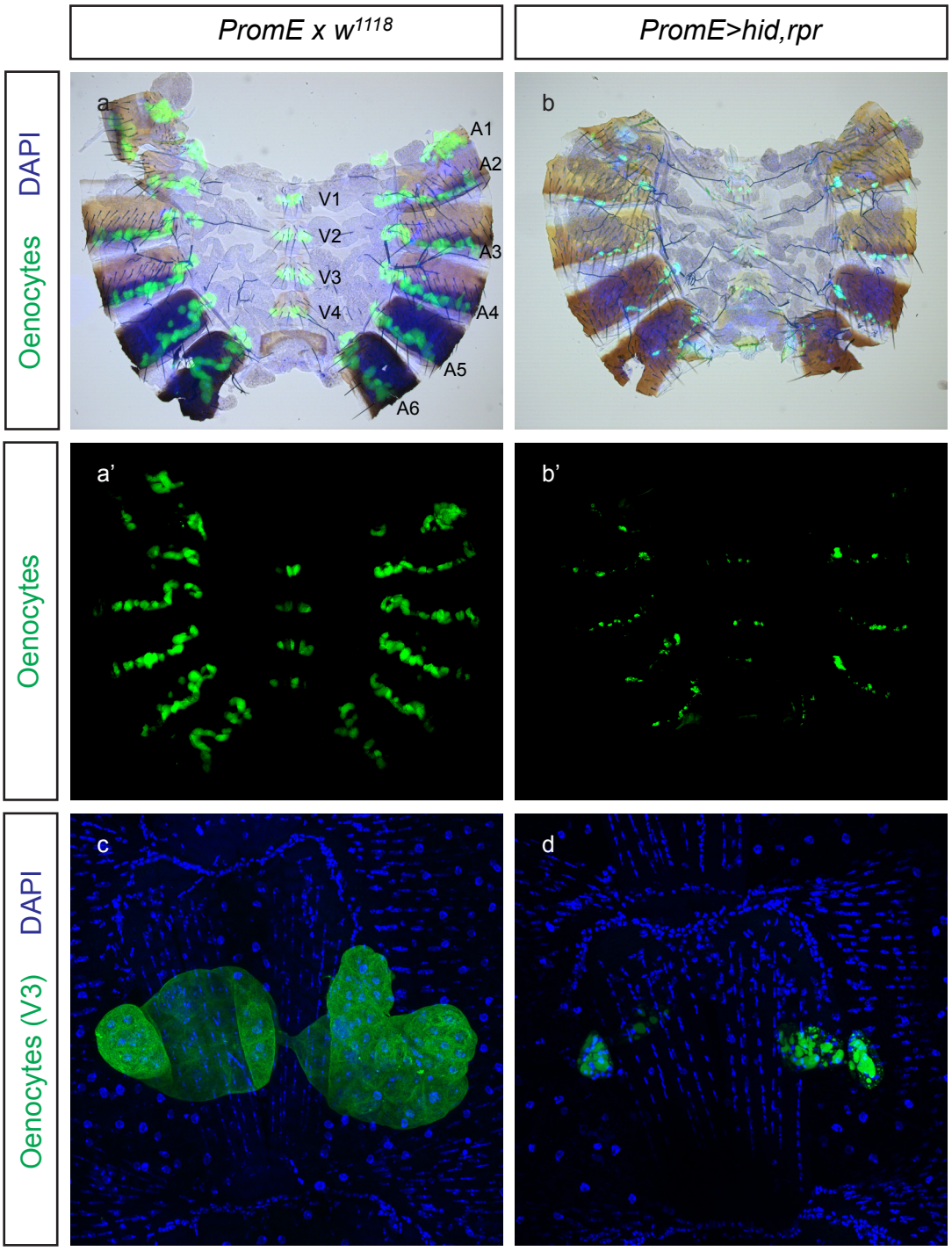
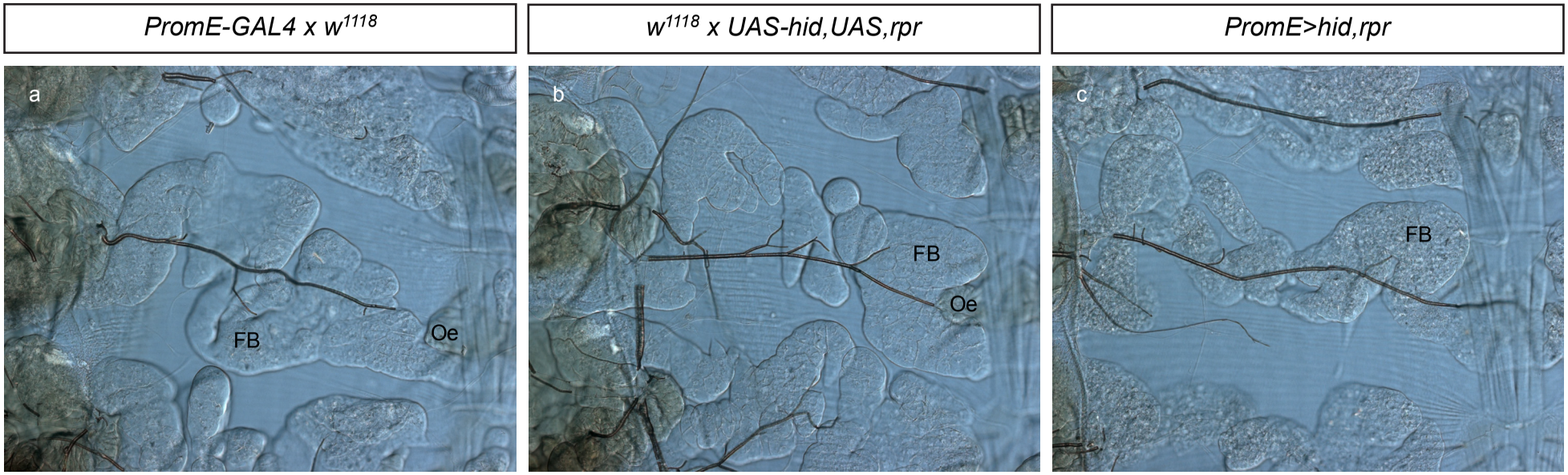

PromE x w1118
PromE>hid,rpr
Oenocytes DAPI
a
A1
A2
V1
V2
A3
V3
V4
A4
A5
A6
b
Oenocytes
a'
b'
Oenocytes (V3) DAPI
c
d

Figure 5.2 Oenocyte ablation does not result in tracheal defects at the gross anatomical level

Tracheal branching in control and oenocyte-ablated 6-day-old adult flies.
(a-b) In control flies tracheal branches, surrounded by fat body, arise from the spiracle of abdominal segment A3 and extend towards V2.
(c) No defects are observed in the tracheal branching pattern or in tubular morphology in oenocyte-ablated flies.


PromE-GAL4 x w1118
w1118 x UAS-hid,UAS,rpr
PromE>hid,rpr
a
FB
Oe
b
FB
Oe
c
FB

5.4 Oenocytes are the site of Cyp4g1-mediated hydrocarbon synthesis

Adult oenocytes are responsible for the production of cuticular hydrocarbons that have important roles in desiccation resistance and courtship behaviour (reviewed by Makki *et al.* In Press). Hence, partial oenocyte ablation results in strong reductions of most if not all cuticular hydrocarbons (Billeter et al., 2009).

As a first step towards analysis of oenocyte functions in metabolism, I attempted to replicate these results using my more efficient ablation protocol. Control and oenocyte-abated flies, as described in section 5.1, were kept on 2x food from 0-6 days prior to hydrocarbon extraction. For this, whole flies were dipped in hexane for 5 minutes, which was subsequently analysed for hydrocarbons using GC-MS. Chromatogram traces of cuticular hydrocarbons show that oenocyte ablation results in the loss of all hydrocarbon peaks (Fig. 5.3a-d). This is therefore consistent with the previous study (Billeter et al., 2009). Together with the confocal data described in section 5.1, the absence of hydrocarbon peaks in experimental flies indicates that our ablation protocol is not only efficient in ablating oenocytes but also in knocking down their function in hydrocarbon synthesis. It will be interesting to test whether internal hydrocarbon species, thought to be stored in the fat body, are similarly affected upon oenocyte ablation.

Again to replicate a previous study, I sought to establish that knockdown of the P450 enzyme *Cyp4g1* also leads to the loss of cuticular hydrocarbon peaks (Qiu et al., 2012). *Cyp4g1* together with its redox partner *cytochrome P450 reductase (Cpr)*, act in the last step of hydrocarbon synthesis, converting LCFA-derived aldehydes into hydrocarbons. Cuticular hydrocarbon profiles were again determined at 6 days for control and experimental (*PromE-GAL4,tub-G80^{ts},UAS-CD8::GFP* also carrying a *UAS-Cyp4g1 RNAi transgene*) flies. I find that although oenocyte-specific *Cyp4g1* knockdown resulted in the loss of most hydrocarbon peaks, two hydrocarbon peaks are still present (Fig. 5.4a-c). This contrasts with

oenocyte ablation and suggests that *Cyp4g1/Cpr* are required for the synthesis of most but not all cuticular hydrocarbons. The identity of these peaks is not certain but GC-MS indicates that they only contain hydrogen and carbon and are therefore hydrocarbons. It will be interesting to explore the origin of the two *Cyp4g1*-independent hydrocarbon peaks, perhaps testing whether they require the LCFA synthetic pathway using Kar RNAi.

Figure 5.3 Oenocyte ablation results in the loss of most, if not all, cuticular hydrocarbons

GC chromatogram traces of cuticular hydrocarbons. IS – Internal standard.
(a-b) Cuticular hydrocarbon traces from control 6-day-old adult male flies.
(c) Oenocyte ablation results in the loss of cuticular hydrocarbons.
(d) Blank samples without flies were processed the same way as the rest of the samples.

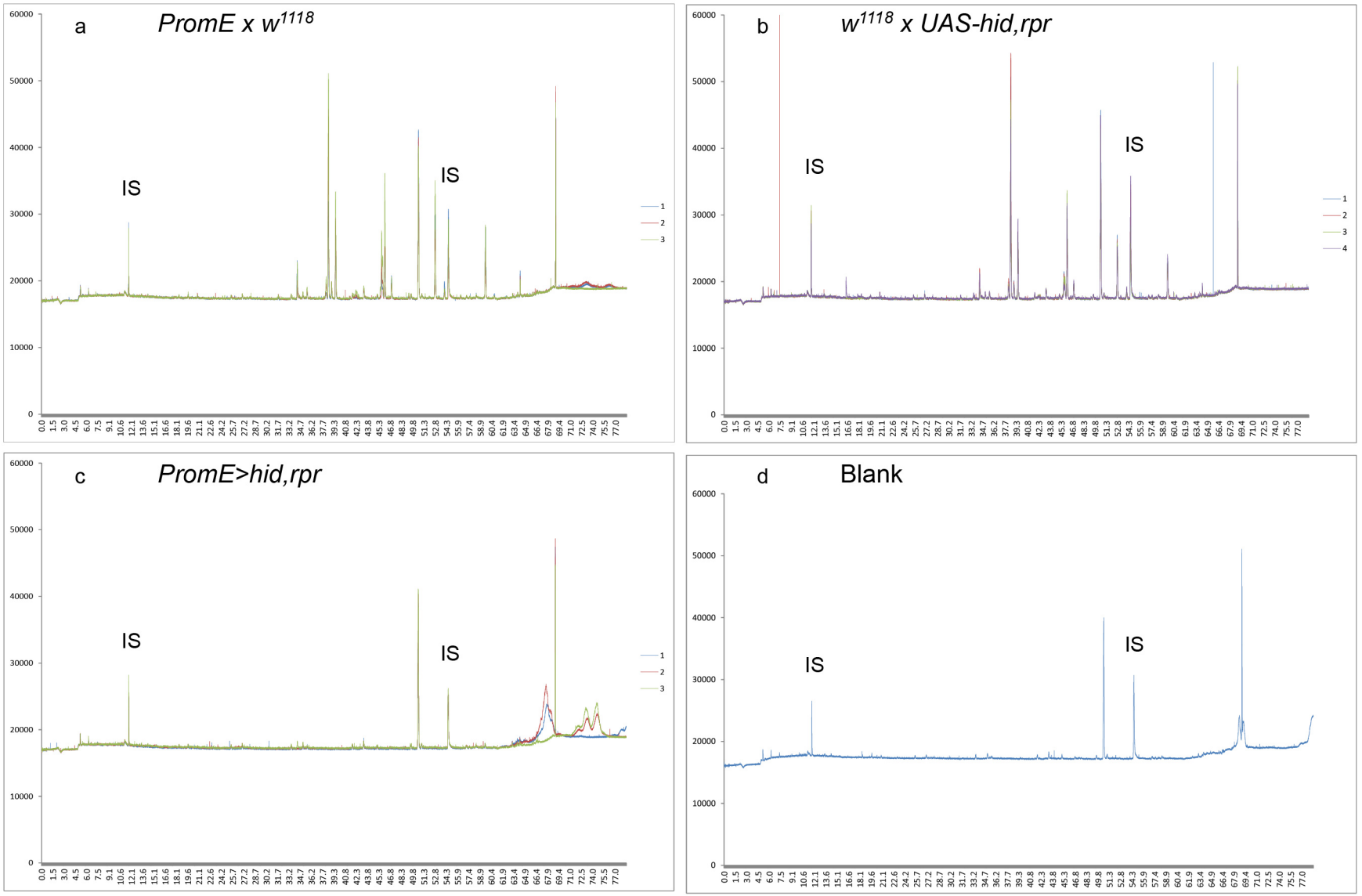
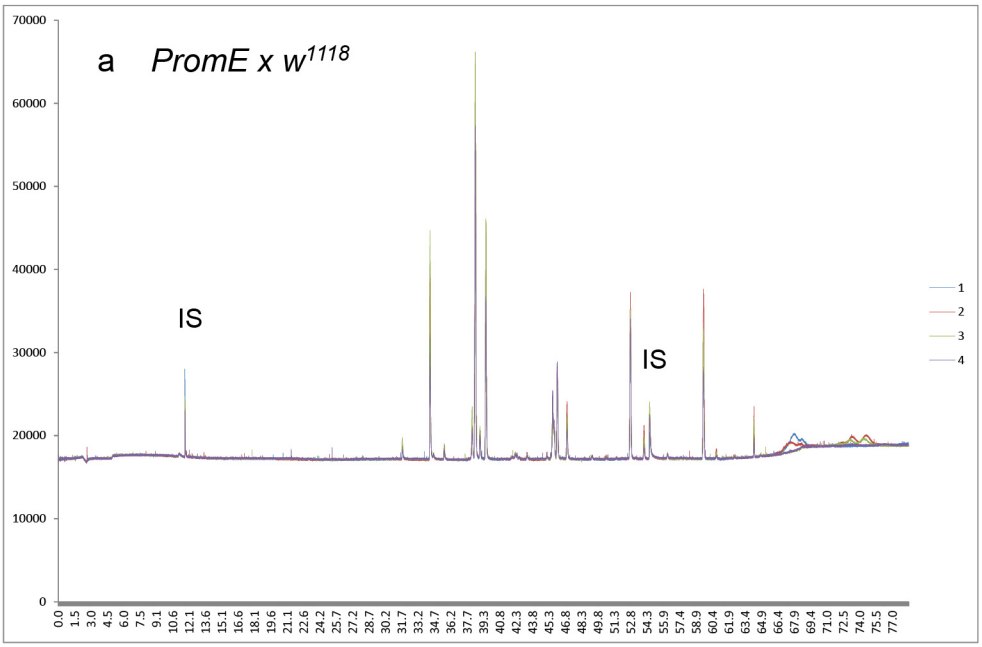
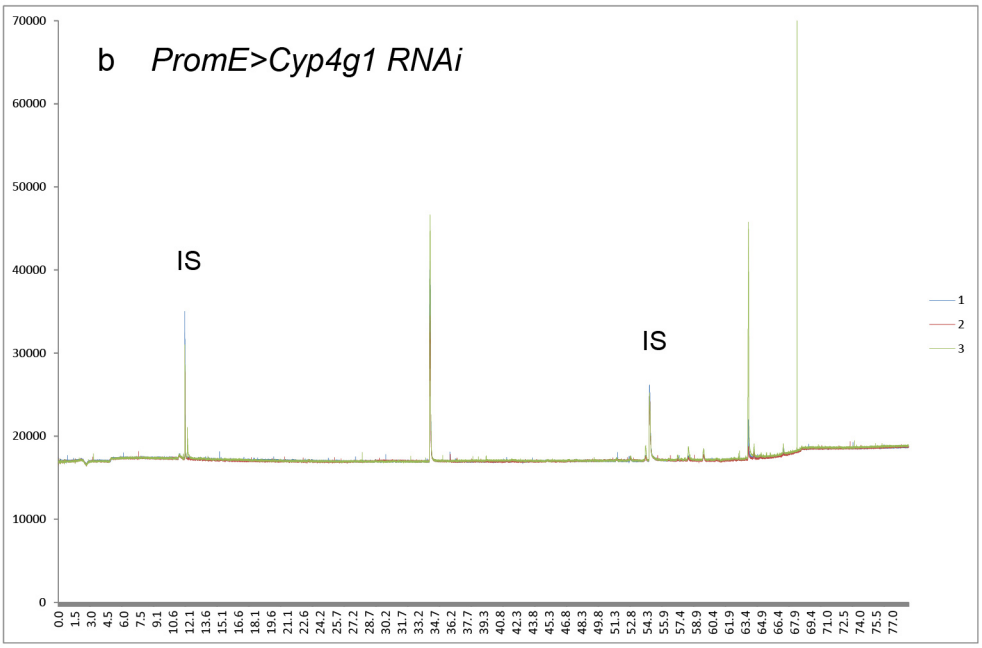
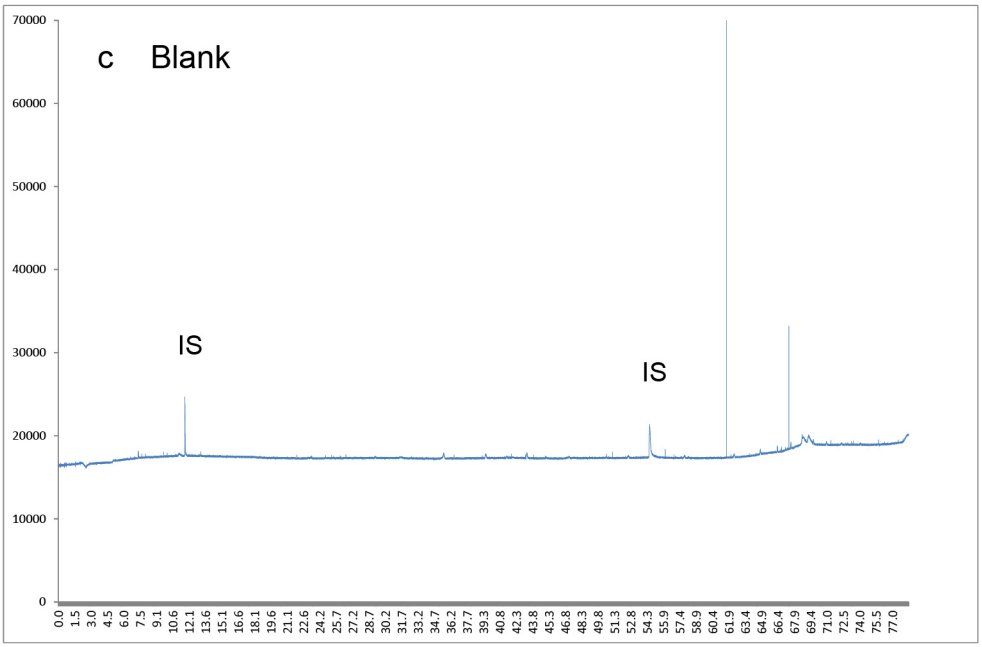

a
PromE x w1118
IS
IS
b
w1118 x UAS-hid,rpr
IS
IS
c
PromE>hid,rpr
IS
IS
d
Blank
IS
IS

Figure 5.4 *Cyp4g1* knockdown results in the loss of most cuticular hydrocarbons

GC chromatogram traces of cuticular hydrocarbons. IS – Internal standard.
(a-b) Cuticular hydrocarbon traces from control 6-day-old adult male flies.
(c) *Cyp4g1* knockdown results in the loss of most cuticular hydrocarbons.
(d) Blank samples without flies were processed the same way as the rest of the samples.


a PromE x w1118
IS
IS
1
2
3
4


b PromE>Cyp4g1 RNAi
IS
IS
1
2
3


c Blank
IS
IS

5.5 *Cyp4g1* knockdown leads to ectopic lipid droplet accumulation in the oenocytes of young flies

Having validated the oenocyte ablation system thoroughly, I was now in a position to analyse possible additional functions of oenocytes in lipid metabolism. Adult flies expressing *UAS-Cyp4g1 RNAi* in oenocytes were dissected at 6 and 14 days of age to look for changes in lipid content. I first observed that lipid droplets are largely absent from the oenocytes of control flies at both timepoints (Fig. 5.5a,a'c,c'). However, the oenocytes of the experimental genotype show a striking accumulation of small lipid droplets (Fig. 5.5b,b',d,d'). Furthermore, at 14 days, oenocytes of the experimental genotype exhibited a punctate CD8::GFP localisation pattern, compared to the more homogeneous expression observed in the controls (Fig. 5.5c-d'). These results demonstrate that *Cyp4g1* is required to suppress oenocyte lipid droplets. This suggests that there may be an interconnection between LDs and hydrocarbon synthesis.

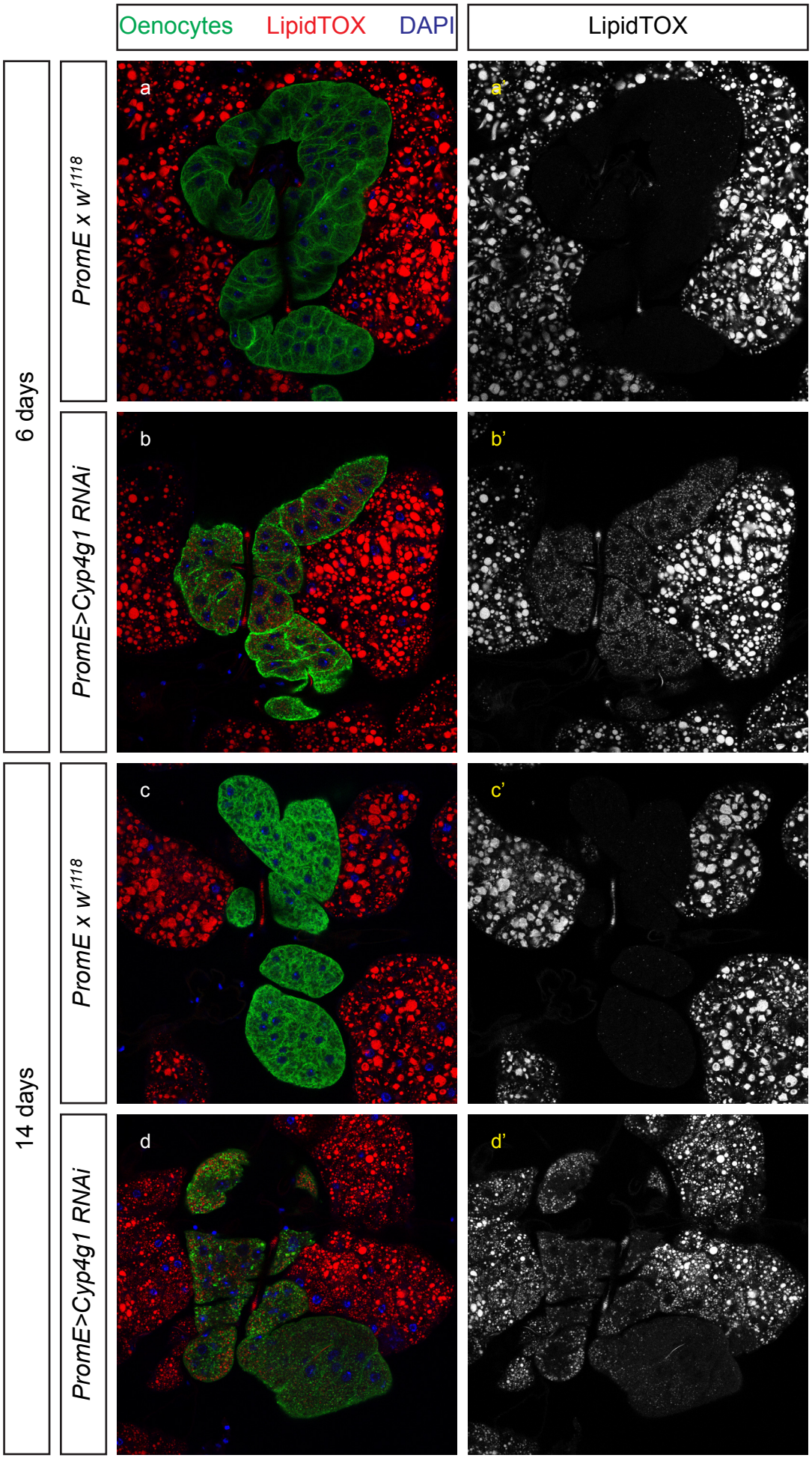
This chapter optimized an efficient oenocyte ablation method and validated its use by replicating the published effect on loss of cuticular hydrocarbons. In addition, I showed that adult oenocytes play an essential role during adulthood and that, unlike larval oenocytes, this appears to be independent of tracheal integrity. My results also support other previously published data, demonstrating that adult oenocytes regulate hydrocarbon synthesis in a *Cyp4g1*-dependent manner. I take this further by showing that impaired hydrocarbon synthesis correlates with lipid droplet accumulation in oenocytes, suggesting that these two types of lipids are in communication with each other. At present, the lipid source for hydrocarbon synthesis is still largely unknown, as is the uptake mechanism into oenocytes. This chapter raises the possibility that hydrocarbon synthesis in oenocytes could affect neutral lipids such as TAGs in other tissues as well. This idea is further explored in chapter 6.

Figure 5.5 *Cyp4g1* knockdown results in lipid droplet accumulation in oenocytes

Oenocytes (*PromE-GAL4,tubG80^{ts},UAS-CD8::GFP*) and fat body are stained with LipidTOX (red).

(a,a',c,c') Lipid droplets are not present in both 6 and 14-day-old control flies.

(b,b',d,d') *Cyp4g1* knockdown results in oenocyte lipid droplet accumulation in both 6 and 14-day old flies.


Oenocytes LipidTOX DAPI
LipidTOX
6 days
14 days
PromE x w1118
PromE>Cyp4g1 RNAi
PromE x w1118
PromE>Cyp4g1 RNAi
a
a'
b
b'
c
c'
d
d'

CHAPTER SIX

Identification of an adult fat body-oenocyte axis

CHAPTER SIX: Identification of an adult fat body-oenocyte axis

6.1 Oenocytes are required to prevent excess fat body TAG

In the preceding chapters, I showed that adult oenocytes are responsive to dietary nutrients, in particular methionine, and that they utilize an LpR1-dependent lipid uptake mechanism. This suggested that adult oenocytes might also interact with the major lipid storage/release organ of the adult fly; the fat body. To test directly whether or not adult oenocytes regulate lipid metabolism in the fat body, I stained abdominal preps from control and oenocyte-ablated 6-day-old flies with LipidTOX. Previous work in the lab indicates that the adult fat body is divided into anatomically distinguishable subtypes, based on location. Subcuticular fat body represents the majority of adipose tissue. Nevertheless a distinct group of adipocytes are associated with the gut at the boundary between midgut and hindgut (I. Stefana and A. Gould, unpublished data). We have named this perivisceral fat body. Using confocal microscopy, I observed that both subcuticular and perivisceral fat body from oenocyte-ablated flies contained lipid droplets that were dramatically increased in size compared to the controls (Fig. 6.1a-f)). To address whether this altered lipid droplet morphology correlates with an increase in stored fat, total TAG levels were measured using GC-MS. Oenocyte-ablated flies showed a dramatic 2.5 fold increase in total TAG levels compared to controls (n=3, $p<0.0001$) (Fig. 6.1g). Together, these results demonstrate that adult oenocytes inhibit excessive fat body TAG storage. Interestingly, I also observed a 1.6 fold increase in the ratio of C16:1/C16:0 (n=3, $p<0.0001$), a proxy for fatty acid Δ9 desaturase activity (Fig. 6.1h). Δ9 desaturase requires oxygen as a cofactor (Kamphorst et al., 2013; Seifried and Gaylor, 1976). This makes it unlikely that oenocyte-ablated adults are suffering from hypoxia, underscoring the tracheal analysis in section 5.3.

Figure 6.1 Oenocyte ablation results in increased fat body TAG content

Both subcuticular and perivisceral fat body is stained with LipidTOX (red). Gut muscles are marked with phalloidin (green).

(a-b) Lipid droplets (red) from the subcuticular fat body of control flies.

(c) Oenocyte ablation results in a dramatic increase in subcuticular fat body lipid droplet size.

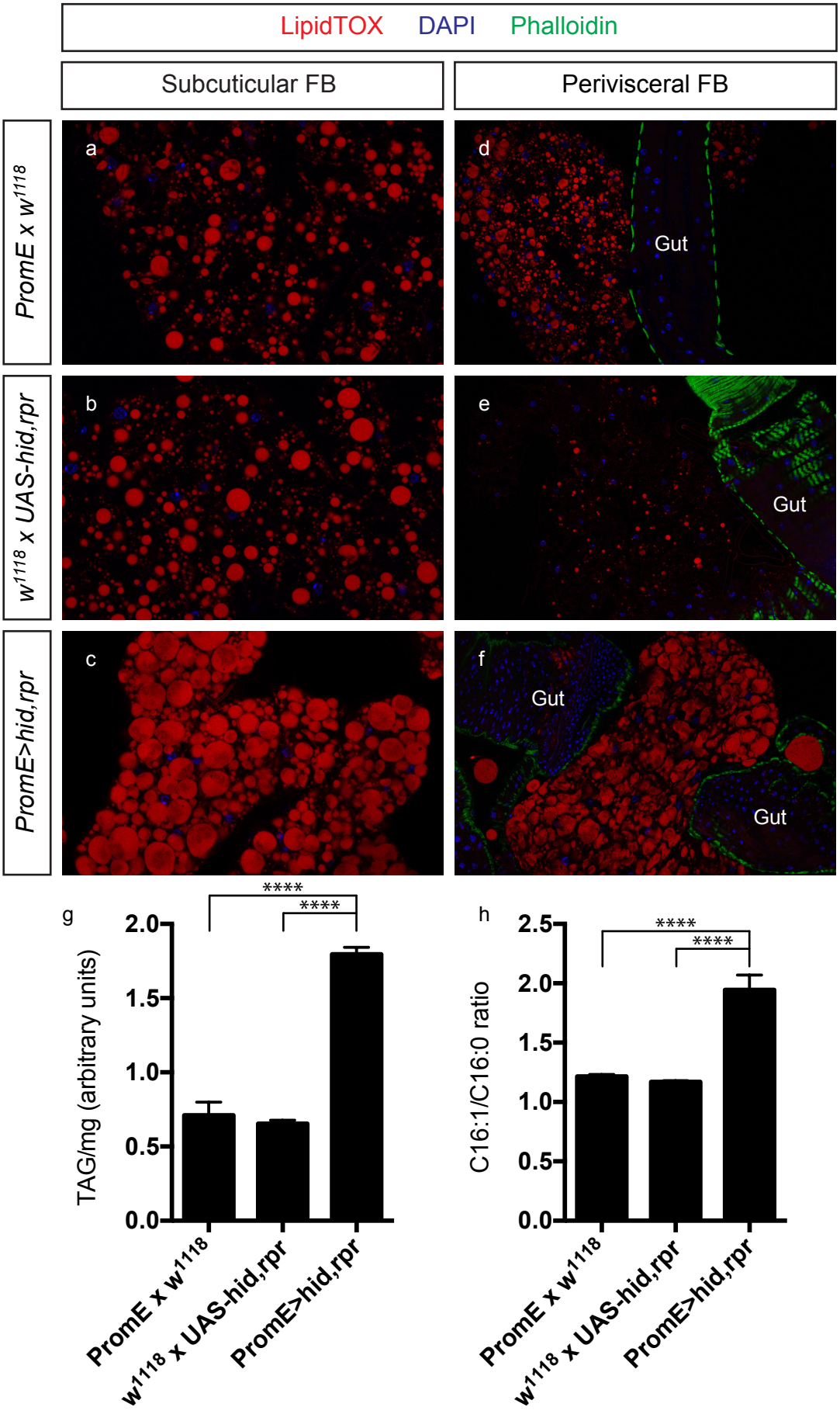
(d-e) Lipid droplets (red) from perivisceral fat body surrounding the gut of control flies.

(f) Oenocyte ablation results in a dramatic increase in perivisceral fat body lipid droplet size.

(g) Whole-animal TAG content is increased 2.5 fold in oenocyte-ablated flies.

(h) The C16:1/C16:0 ratio is increased 1.6 fold in oenocyte ablated flies.

(**** $p<0.0001$)


LipidTOX
DAPI
Phalloidin
Subcuticular FB
Perivisceral FB
PromE x w1118
w1118 x UAS-hid,rpr
PromE>hid,rpr
a
b
c
d
e
f
Gut
Gut
Gut
Gut
g
TAG/mg (arbitrary units)
2.0
1.5
1.0
0.5
0.0
****
****
PromE x w1118
w1118 x UAS-hid,rpr
PromE>hid,rpr
h
C16:1/C16:0 ratio
2.5
2.0
1.5
1.0
0.5
0.0
****
****
PromE x w1118
w1118 x UAS-hid,rpr
PromE>hid,rpr

6.2 The fat body regulates oenocyte lipid metabolism

I next addressed whether or not there is reciprocal regulation from fat body to oenocyte. The approach was to manipulate a series of lipid metabolic genes in the fat body, using a fat body-specific GAL4 line (*Lpp-GAL4,tub-G80^{ts}*), in each case looking for changes in oenocyte lipid content. Firstly, lipid mobilization was increased from the fat body by overexpressing *brummer.* Abdominal preps from experimental and control flies were stained with LipidTOX. Fat body from *Lpp>bmm* flies showed a dramatic change in subcellular lipid droplet localisation. Unlike controls, lipid droplets were fewer in number and were observed in a perinuclear pattern rather than throughout the fat body adipocyte. However, I did not observe lipid droplet accumulation in adult oenocytes (Fig. 6.2a-b'). These results demonstrate that increasing lipase activity in fat body cells triggers changes in lipid droplet cytosolic localisation that may or may not be consistent with increased lipolysis. Furthermore, it strongly suggests that increased lipid mobilisation from the fat body does not result in lipid accumulation within oenocytes. Both findings contrast with larvae, where increasing Bmm in fat body leads to lipid droplet accumulation in oenocytes but lipid droplet loss without perinuclear redistribution in fat body. It is possible that larval and adult fat body cells do not respond to increased Bmm in the same way and/or that there are significant differences in the oenocyte response to increased circulating lipids.

Sterol regulatory element-binding protein (SREBP) is a transcription factor that upregulates the expression of several key enzymes involved in lipid synthesis, such as ACC and FAS (Porstmann et al., 2008; Shao and Espenshade, 2012). I found that overexpression of an SREBP dominant-negative construct in the fat body had no obvious effect on fat body lipid droplet size or density as revealed by confocal analysis (Fig. 6.2a,a',c,c'). Whole-animal TAG measurements using GC-MS would be required to confirm that there is no change in lipid storage. Nevertheless, *Lpp>SREBP^{DN}* adults show a consistent increase in lipid droplets in the oenocytes relative to controls (Fig 6.2a,a',c,c'). Importantly, this last result demonstrates that

SREBP is required in the fat body to prevent inappropriate lipid droplet accumulation in oenocytes. One possibility here is that the adult fat body and oenocytes compete for precursors for *de novo* lipogenesis such that reducing fatty acid synthesis in the fat body leaves more precursors for lipid synthesis in the oenocytes. Regardless of the underlying mechanism, these results show that there is metabolic regulation from fat body to oenocytes.

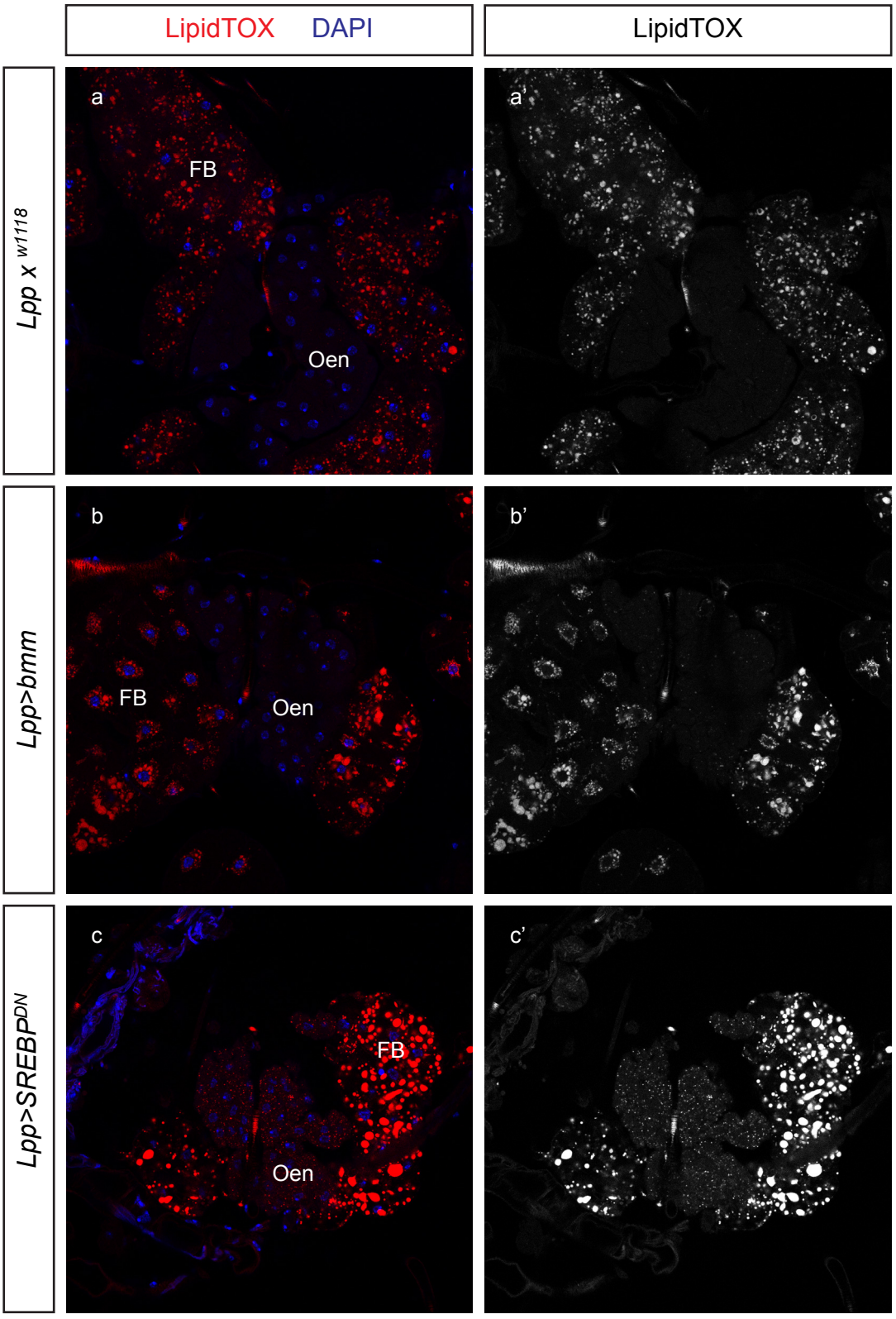
Figure 6.2 SREBP^{DN} overexpression in the fat body results in lipid droplet accumulation in oenocytes

Oenocyte and fat body are stained with LipidTOX (red)

(a) Fat body and oenocytes from control flies.

(b) *Brummer* overexpression in the fat body results in a perinuclear lipid droplet pattern. No lipid droplets are observed in oenocytes.

(c) *SREBP^{DN}* overexpression in the fat body results in lipid droplet accumulation in oencoytes.


LipidTOX DAPI
LipidTOX
Lpp x w1118
Lpp>bmm
Lpp>SREBPDN
a
a'
FB
Oen
b
b'
FB
Oen
c
c'
FB
Oen

6.4 A link between oenocytes and gut lipid metabolism

During the course of conducting many different oenocyte manipulations, I tested the *Drosophila* ortholog of Hepatocyte Nuclear Factor 4, a key regulator of hepatic lipid metabolism. The *Drosophila* genome encodes only one ortholog of HNF4 (dHNF4), which is expressed in oenocytes and other tissues such as the gut (Palanker et al., 2009). Whilst no oenocyte-specific role for dHNF4 has been addressed, dHNF4 mutants revealed an important role for this receptor in regulating lipid metabolism and β-oxidation (Palanker et al., 2009). Using PromE to drive HNF4 RNAi with tubG80^{ts}, larvae were raised at 18^{o}C on 2x food up to eclosion, after which they were transferred to 25^{o}C and maintained on CDD 22. Oenocyte-specific RNAi-mediated knockdown of dHNF4 resulted in a striking cell-autonomous accumulation of lipid droplets (Fig. 6.3a-b'). This shows that inhibiting β-oxidation in oenocytes, leads to lipid droplet accumulation. Guts of the control and experimental genotype (*PromE>dHNF4 RNAi)* were also dissected and stained with LipidTOX. Control guts showed no lipid droplets in the midgut, intriguingly however, guts of the experimental genotype exhibited a striking accumulation of lipid droplets in the posterior midgut (Fig. 6.4a-b'). How impaired β-oxidation in oenocytes might promote lipid droplet accumulation in the midgut is not yet clear but will be discussed further in chapter 7.

Figure 6.3 *HNF4* knockdown in oenocytes results in lipid droplet accumulation

(a) No lipid droplets are observed in control oenocytes.

(b) *HNF4* knockdown results in lipid droplet (red) accumulation.

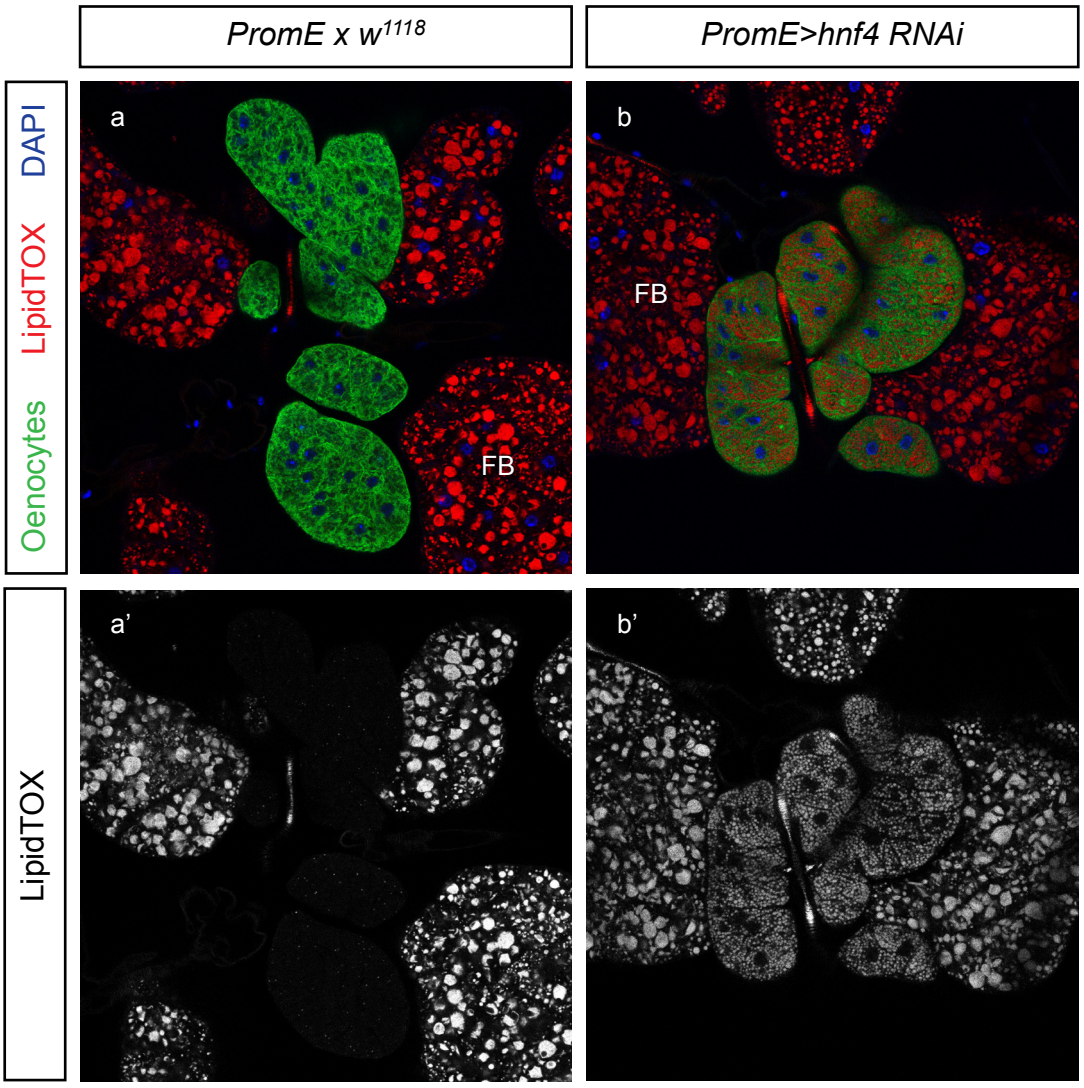

PromE x w1118
PromE>hnf4 RNAi
Oenocytes LipidTOX DAPI
LipidTOX
a
b
FB
FB
a'
b'

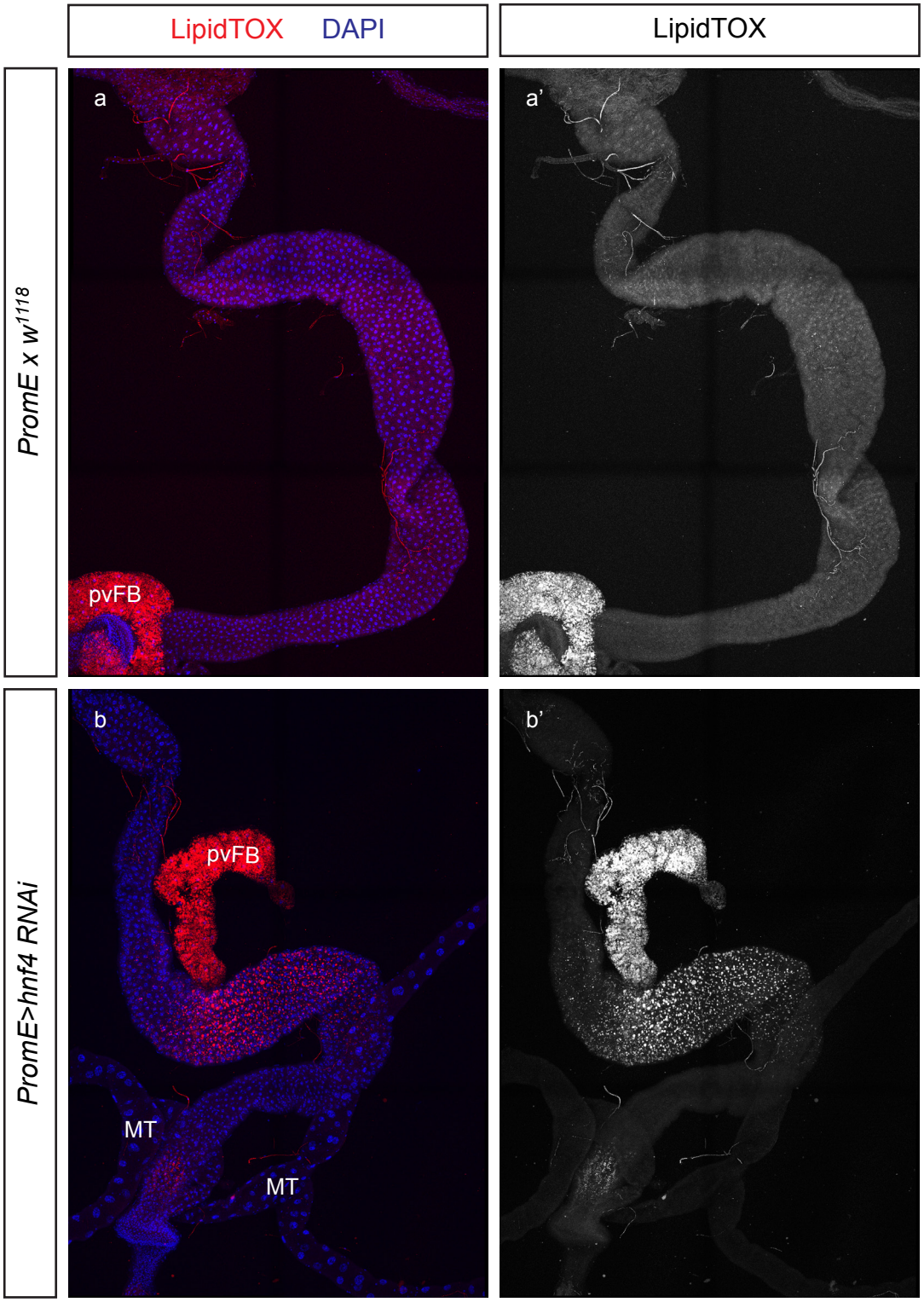
Figure 6.4 *HNF4* knockdown in oenocytes results in lipid droplet accumulation in the midgut

Control and experimental guts stained with LipidTOX (red).

(a-a') No lipid droplets are observed in the guts of control flies.

(b-b') *HNF4* knockdown in oenocytes results in lipid droplet (red) accumulation in the midgut.

pvFB – Perivisceral Fat Body. MT – Malphigian Tubules.


LipidTOX DAPI
LipidTOX
PromE x w1118
PromE>hnf4 RNAi
a
a'
b
b'
pvFB
pvFB
MT
MT

CHAPTER SEVEN

Discussion

CHAPTER SEVEN: Discussion

7.1 Development of a holidic diet for *Drosophila*

This thesis focused on exploring the influence of nutrition on lipid metabolism in adult *Drosophila*. To enable precise control over macro- and micronutrients, I developed two chemically defined diets, using as a protein source either a casein peptide extract (CDD 24) or individual amino acids (CDD 22). Both diets were optimised from the existing recipes of Sang and Rapport (Rapport et al., 1983; Sang, 1956) by replacing dietary 2'(3') nucleoside monophosphates with 5'(3') nucleoside monophosphates. Furthermore, the antifungal agents, nipagin/bavistan, and the antibiotics, penicillin/streptomycin, were also introduced to prevent fungal/bacterial growth on the media. Both chemically defined diets successfully supported the complete *Drosophila* life cycle, from embryo to adult, for multiple generations. The rapid and extensive growth occurring during the larval phase of development was used as stringent assay for the efficacy of chemically defined diets. I found that larval development is completed within ~8 days on CDD 22, ~6 days on CDD 24 and ~4 days on the optimal 2x yeast diet. At least some of the delay on the defined diets may be due to reduced food intake, as I observed that young larvae on CDD 24 and CDD 22 (but not 2x yeast) spend a substantial fraction of their time crawling on the side of the vial, outside the food. The delay between both defined diets (CDD 22 and CDD 24) versus the 2x yeast diet could be because there is a deficiency or imbalance in one or more non-protein components such as fat or a micronutrient. Hence, even though dietary fats are not strictly essential for development, their presence could improve various physiological parameters such as larval growth rate. However, this appears to be unlikely as extensive *de novo* lipogenesis by larvae ensures that their lipid profiles remain very similar on standard versus lipid-free media (Palm et al., 2012). Whatever non-protein component(s) are suboptimal in the defined diets, the larval timing difference between CDD22 and CDD24 does strongly suggest that future gains can be made by further optimization of the

amounts and/or ratios of individual amino acids. One possible approach towards this aim, based on the idea of "you are what you eat", would be to test amino-acid ratios calculated on the basis of those present in the total body of larvae on 2x yeast. Nevertheless, even without additional optimization, the data in this thesis demonstrate that the holidic CDD 22 diet is useful for defining the roles of individual amino acids, such as methionine, in growth and lipid metabolism.

7.2 Adult organs grow and lipid metabolism changes with age

Despite a great deal of research on nutritional and genetic regulation of fly lifespan, relatively little is known about how the physiological properties of internal organs change with age. Experiments in this thesis go some way towards filling this gap in our knowledge. For example, although the body mass of adult male flies is relatively homeostatic during adulthood, I found that this masks the growth of several cell types/organs. It had previously been shown by the Bilder lab that the adult midgut increases in volume in a nutrient-dependent manner during adulthood (O'Brien et al., 2011). My studies have demonstrated that the midgut is not unique and that several other tissues are also capable of adult growth. In summary, the organs tested can be classified into three categories: 1. Those that do not grow during adulthood but require amino acids to maintain volume (e.g. central brain and muscles). 2. Those that require amino acids to grow during adulthood and also to maintin volume (e.g. mNSCs). 3. Those that grow during adulthood in an amino acid dependent manner but can maintain initial volumes without dietary amino acids (e.g. oenocytes and fat body, Fig. 7.1, 7.2).

It is interesting that two adult tissues relevant to lipid metabolism, the fat body and oenocytes, both display growth and other changes during ageing. Given that adult TAG content (I. Stefana and A. Gould, pers. comm.) and fat body lipid droplets both decrease with age it is intriguing that I observed an amino-acid dependent increase in the volume of fat body cells from 0-2 weeks (Fig. 7.2). The basis for the inverse correlation between fat

body cell volume and lipid droplet content is not yet clear but it may be important, if technically challenging, to count the total number/volume of fat body cells per animal. Reduced insulin signalling with age could explain the reduction of fat body lipid droplets, although not the increased cell volume. Consistent with this, insulin signalling has been shown to promote increases in both TAG content and lipid droplet size in the adult fat body (DiAngelo and Birnbaum, 2009). Insulin signalling also positively regulates cell number in the adult fat body but with no profound effect on cell size (DiAngelo and Birnbaum, 2009). This published study, together with my own results, suggest that amino acids may stimulate fat body cell growth independently of the insulin pathway, possibly via a TOR-dependent mechanism. It is also possible that the reduction in fat body lipid droplets, and perhaps other age-related physiological changes, reflect decreased feeding with age. To test this hypothesis, food intake and feeding behaviour on 2x yeast and CDD22 could be quantified with age using various assays such as food dye incorporation, proboscis extension and capillary feeding (CAFE) assay (Al-Anzi et al., 2010; Ja et al., 2007; Wong et al., 2008; Wong et al., 2009).

The average size of lipid droplets in the fat body increases on CDD 22-Met without an increase in total body TAG. As the fat body stores the majority of total TAG, this suggests that, directly or indirectly, dietary methionine leads to the redistribution of TAG among fewer but larger lipid droplets. Increases in lipid droplet size have also been observed in mammalian and *Drosophila* cell lines upon inhibition of CDP-choline-dependent PC synthesis, demonstrating that this phospholipid prevents inappropriate coalescence of lipid droplets (Krahmer et al., 2011). This raises the possibility that flies also express the appropriate enzymes for methionine/PEMT-dependent PC synthesis, which in turn would regulate lipid droplet size. To date however, PEMTs have not been identified in insects (Gibellini and Smith), although this may warrant further investigation as PEMT-orthologues have been discovered in *C.elegans* (Brendza et al., 2007; Palavalli et al., 2006).

Another surprising finding from the organ growth experiments was that there is a 3.7 fold increase in oenocyte cell volume from 0 to 2 weeks of age. Although this occurs without any detectable replication of the nuclear genome, it is dependent upon PI3K signalling and so presumably represents a *bona fide* anabolic growth process rather than some kind of osmotic swelling. Given their large nuclear size and strong DAPI staining, adult oenocytes may be polyploid. If this is the case, the well-known correlation between nuclear ploidy and cell size in larvae may be broken by adult oenocytes. Concomitant with adult oenocyte growth during ageing, there is an increase in intracellular lipid droplets (Fig. 7.2). Inhibiting the TOR pathway also promotes oenocyte lipid droplet accumulation at young ages, suggesting that the TOR pathway may be less active in old versus young oenocytes. It is important to highlight that, during adult ageing, there is a reciprocal correlation between the lipid droplet content of fat body cells and oenocytes: the former decreases while the latter increases. The mechanistic basis for this correlation does not appear to be as simple as for the larval fat body-oenocyte axis (Gutierrez et al., 2007), as increasing adult fat body lipolysis (*Lpp>Bmm*) was not sufficient to induce lipid droplet accumulation in adult oenocytes. This suggests that adult *Drosophila* oenocytes may not be passive recipients of haemolymph lipids, as thought for some mammalian tissues in the "lipid overflow hypothesis" (Sozio et al., 2010).

I also found that, with increasing age, oenocytes accumulate the brown pigmentation and autofluorescence that is characteristic of lipofuscin (Seehafer and Pearce, 2006; Terman and Brunk, 2004). In other species, lipofuscin is used as a marker of ageing and it will be interesting to see in *Drosophila* whether the increase with chronological age is specific to oenocytes or also found in other tissues, such as the fat body. In addition, for lipofuscin to be a true marker of the fly ageing process and not just of chronological age, its rate of accumulation should be compared between wild-type flies and mutants with extended lifespan.

7.3 Adult oenocytes are nutrient responsive

I found that young adult male flies kept for 2 weeks on CDD 22-Met, but not on the control CDD 22 diet, accumulate lipid droplets in their oenocytes. Previous studies have shown that a dietary methionine deficiency in rodents specifically promotes steatosis in hepatocytes (Basaranoglu et al., 2010; Koteish and Diehl, 2001). This raises the possibility that certain aspects of nutritional/metabolic regulation are shared between adult oenocytes and hepatocytes. To investigate the mechanism by which lipid droplets accumulate in young adult oenocytes on CDD 22-Met, a number of genetic manipulaitons were performed. *PromE>SAM-S RNAi* flies on CDD 22 showed no striking differences in oenocyte number or morphology compared to the controls. However, I found that *SAM-S* knockdown coupled with dietary methionine deficiency results in a partial oenocyte-ablation phenotype. It is possible that combining both manipulations blocks flux through the one carbon metabolism pathway, thereby resulting in oenocyte loss.

Inhibiting the TOR pathway also promotes oenocyte lipid droplet accumulation suggesting that the TOR pathway may be less active on CDD 22-Met. This could be mediated through increased AMPK signalling which has been shown to be activated in mammalian hepatocytes following a decrease in intracellular SAM (Lu and Mato, 2008; Martinez-Chantar et al., 2006; Mihaylova and Shaw, 2011; Vazquez-Chantada et al., 2009).

This thesis also showed that oenocyte-specific knockdown of *LpR1* blocks lipid droplet accumulation, both on CDD 22 and CDD 22-Met. This demonstrates that oenocytes take up lipids under both physiological and steatosis-promoting conditions (Fig. 7.2). Hence, lipid uptake from the haemolymph may account for the increased oenocyte lipid droplet accumulation that is observed with age. In mammals it has been demonstrated that a methionine deficient diet inhibits PEMT-dependent PC synthesis in the liver, which results in impaired VLDL assembly and secretion, thereby blocking lipid export and promoting steatosis. Impaired lipid export is unlikely to account for oenocyte steatosis as in insects the fat body is the principal site of lipophorin synthesis (Palm et al., 2012),

although it has not been directly tested whether oenocytes express any of the apolipophorin genes. Furthermore, unlike mammals, the primary phospholipid present in insect lipophorin is the PEMT-independent phospholipid; phosphatidylethanolamine (Palm et al., 2012). This strongly suggests that lipophorin assembly is not affected when methionine is removed from the diet.

7.4 Links between oenocyte lipid droplets and hydrocarbons

Despite much research on hydrocarbons, it is unclear to what extent *de novo* lipogenesis or fatty acid uptake contribute to the hydrocarbon-precursor fatty acid pool. Both larval and adult oenocytes synthesise VLFCA via the same Acc/Kar-dependent pathway. In larvae, these VLCFAs act as a remote signal to waterproof the tracheal system, whereas in adults they undergo *Cyp4g1*-dependent oxidative decarbonylation to generate hydrocarbons (Parvy et al., 2012; Qiu et al., 2012). Whole extract analysis has revealed that larval oenocytes also synthesise hydrocarbons (R. Makki and A. Gould, unpublished data), however it is not clear whether they are deposited on the cuticle in the same way as in adults. In other insects it has been shown that hydrocarbons synthesised in the oenocytes can be transported via lipophorins to other other tissues such as the fat body (reviewed in Blomquist and Bagnères, 2010). It has not been clear whether the fat body acts as a store for hydrocarbons, or whether the hydrocarbons are able to regulate fat body lipid metabolism.

Even though adult oenocyte-specific *LpR1* knockdown blocks lipid droplet accumulation, preliminary data indicates that the cuticular hydrocarbon profile is not affected. This suggests that either *de novo* fatty acid synthesis by oenocytes serves the fatty acid requirement for hydrocarbons, or that certain fatty acids dedicated to hydrocarbon production are taken up from the haemolymph by an alternative mechanism, perhaps involving *LpR2*. Indeed, *LpR2* not *LpR1* appears to be required for lipid uptake in larval oenocytes (Parvy et al., 2012). Hydrocarbons produced in oenocytes are derived from fatty acids which are

desaturated, elongated and then subjected to oxidative decarbonylation to produce hydrocarbons (Billeter et al., 2009; Qiu et al., 2012). Aldehyde oxidative decarbonylation is catalysed by *Cyp4g1* and its redox partner *Cpr*, both of which are strongly expressed in oenocytes. Towards better understanding oenocyte function, I initially replicated previous results by demonstrating that oenocyte ablation or oenocyte-specific *Cyp4g1* knockdown results in a strong reduction of most cuticular hydrocarbons. I take these published findings further by showing that *Cyp4g1* knockdown flies exhibit an increase in oenocyte lipid droplet accumulation. This suggests that fatty acids, which would normally be converted to hydrocarbons, are instead stored as TAG, which would imply that these two lipid pools are interconnected (Fig. 7.1, 7.2).

7.6 An adult fat body-oenocyte axis

Ablation of the adult oenocytes results in increased fat body lipid droplets and a concomitant ~2.5 fold increase in total body TAG. Even though I cannot rule out lipid metabolic effects mediated by leaky *PromE-GAL4* expression in the ejaculatory bulb (Billeter et al., 2009), these results strongly suggest that adult oenocytes have important roles in regulating systemic metabolism relevant to fat body TAG storage and energy homeostasis, not just to cuticular hydrocarbons (Fig. 7.2). This effect could be mediated by an oenocyte-derived signal, acting on the fat body to prevent lipogenesis, or to stimulate lipolysis. In favour of the latter possiblity, oenocyte-ablated larvae display reduced TAG mobilisation upon starvation (Gutierrez et al., 2007). A similar signal could be produced by adult oenocytes which acts even in the fed state to promote lipolysis. Further evidence that adult oenocytes are able to regulate lipid metabolism is provided by a genome-wide obesity screen, which revealed that several lipid metabolic genes, including glycerol kinase and a fatty acid elongase, induce marked changes in total triglyceride when they are knocked down in oenocytes (Pospisilik et al., 2010). My results together with this study strongly suggest that oenocytes regulate fat body-stored TAG.

The 1.7 fold increase in the desaturation index (C16:1/C16:0 ratio) in TAG observed in oenocyte-ablated adults suggests increased activity of Δ9 desaturase. This enzyme requires oxygen as a cofactor (Kamphorst et al., 2013; Seifried and Gaylor, 1976), making it unlikely that oenocyteless adults are hypoxic. In support of this I observe no tracheal defects at the gross anantomical level in oenocyte-ablated flies. This is in contrast to oenocyte-ablated larvae which show air filling tracheal defects and transcriptional upregulation of the *charybdis* and *scylla* hypoxic-responsive genes (Parvy et al., 2012). Together, these results suggest that the excess lipid accumulation in oenocyte-ablated adults is unlikely to be a result of hypoxia-driven impaired β-oxidation. To confirm this, hypoxia reporters and whole-animal lactate measurements could be used. Interestingly, an increased desaturation index (C18:1/C18:0 ratio) was also observed in whole TAG in *Cyp4g1* mutant larvae, demonstrating that these larvae, unlike oenocyteless larvae, are not hypoxic (Gutierrez et al., 2007). Thus, despite complications with the larval oenocyteless phenotype, it appears that both larval and adult oenocytes regulate whole body TAG composition. This is consistent with oenocytes playing a critical role in regulating lipid metabolism in a systemic manner.

In this thesis I show that overexpression of $SREBP^{DN}$ in the fat body leads to lipid droplet accumulation in the oenocytes, indicating that the fat body is able to regulate oenocyte lipid metabolism (Fig. 7.1, 7.2). One possible explanation for this effect is that both the oenocytes and fat body compete for *de novo* lipogenesis precursors, such that reducing fatty acid synthesis in the fat body leaves more precursors for lipid synthesis in the oenocytes. Interestingly, in larvae, the fat body also regulates oenocyte lipid metabolism (Gutierrez et al., 2007). Lipid accumulation can be induced by stimulating fat body lipid mobilisation, either through starvation or by overexpressing the lipase *brummer* in the fat body. In contrast, overexpressing *brummer* in the adult fat body does not lead to oenocyte lipid droplet accumulation. The much lower TAG levels present in adult flies as compared to L3 larvae might contribute to this difference. It is still inconclusive whether starvation induces lipid droplet accumulation in adult

oenocytes and methionine diets have yet to be tested with respect to larvae. Thus, even though larval and adult oenocytes are both responsive to dietary nutrients and both communicate with fat body, it is not yet clear how similar the underlying mechanisms are. Nevertheless, taken together, the results in this thesis demonstrate clearly that there is bidirectional regulation of lipid metabolism between the oenocytes and fat body during adulthood.

My results suggest that oenocytes may not only regulate the fat body but also the midgut . Oenocyte-specific knockdown of *dHNF4* induces cell autonomous lipid droplet accumulation. As *dHNF4* has been shown to positively regulate β-oxidation (Palanker et al., 2009), this effect could result from impaired lipid catabolism within oenocytes. Perhaps more interestingly, *dHNF4* knockdown in oenocytes also appears to promote lipid droplet accumulation in the midgut of adult male flies, raising the possibility that oenocytes not only interact with the fat body but also with other lipid handling tissues such as the gut (Fig. 7.1, 7.2). As the adult flies are kept on the fat-free CDD 22, it is likely that these lipid droplets are a result of increased midgut *de novo* lipogenesis, as has been shown previously (Palm et al., 2012). This study has shown that LTP is required to transfer DAG and sterols from the larval gut on lipophorin particles for transport to other tissues. Knockdown of either LTP or Lpp results in a dramatic accumulation of lipids in the gut, similar to what I observe after *dHNF* knockdown in oenocytes (Palm et al., 2012). Although it remains to be tested, I propose a working model whereby certain lipids are transported directly from the gut to the oenocytes (Fig. 7.2). Impaired β-oxidation within oenocytes would inhibit further lipid uptake, resulting in a backlog of unprocessed lipids within the midgut.

7.7 Concluding remarks

In this thesis I have developed and synthesised an amino acid-defined holidic diet that allows the manipulation of specific nutrients. Furthermore, I have also identified useful physiological makers of age, including changes in lipid metabolism, which can be used to study age-dependent changes in

metabolism. Together with these markers, I also show that adult oenocytes, previously shown to be involved in hydrocarbon synthesis, grow in an amino acid/TOR-PI3K dependent manner without replication of the nuclear genome.

Using the holidic diet, I demonstrate that dietary methionine deficiency mediates Lipophorin receptor 1 mediated lipid accumulation in oenocytes, in a manner akin to that described for the mammalian liver, raising the possibility that certain aspects of metabolic regulation are shared between these two organs.

Genetic oenocyte-ablation results in increased fat body TAG content. Furthermore, certain fat body manipulations (*Lpp>SREBP*^{DN}) induce oenocyte lipid droplet accumulation. Together these results demonstrate bidirectional metabolic coupling between these two organs. Finally, these results suggest for the first time that oenocytes may regulate gut lipid metabolism.

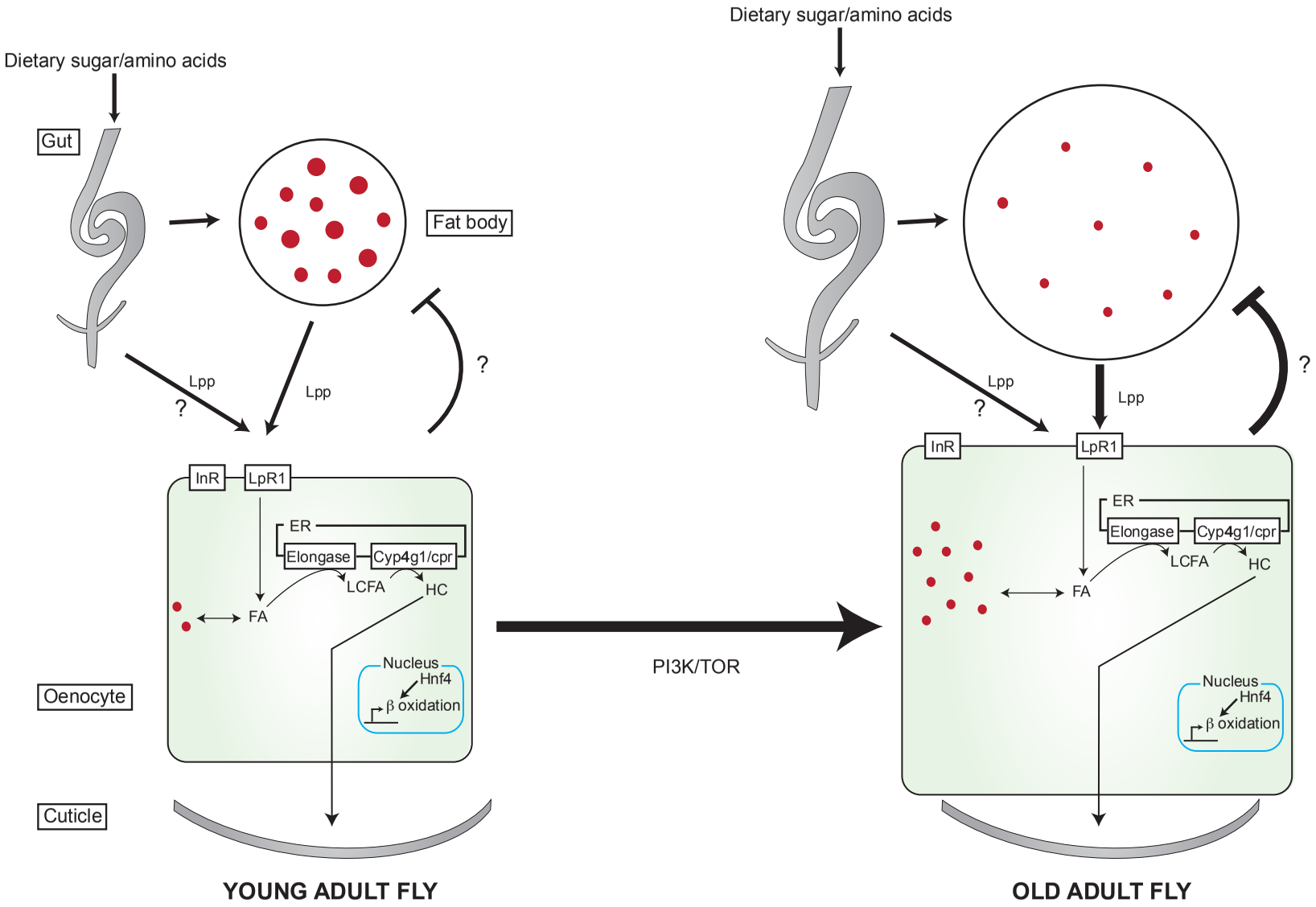
Figure 7.1 Table summarising oenocyte and fat body genetic manipulations

This table summarises the oenocyte-specific (*PromE-GAL4,tub-G80^{ts},UAS-CD8::GFP*) and fat body-specific (*Lpp-GAL4,tub-G80^{ts}*) genetic manipulations carried out in this thesis. For each manipulation, changes in oenocyte lipid droplet content and volume were assayed, as were lipid droplet changes in both the fat body and gut. GC-MS was used to examine for changes in cuticular hydrocarbons. Finally, any changes in CD8::GFP expression were also noted.

Manipulation	Function	Oenocyte		FB	Gut	Hydrocarbons	CD8 GFP
		Lipid droplet accum.	Volume	TAG content	Lipid droplet accum.		Affected?
PromE>LpR1 RNAi	Lipid uptake	-	ND	↑	-	ND	-
PromE>Cyp4g1 RNAi	Oxidative decarbonylation	↑	-	↓	-	Reduction in the majority of peaks	+
PromE>Tsc1+2	TOR/PI3K	↑	↓	ND	-	ND	-
PromE>Rheb	TOR/PI3K	-	↑	ND	-	ND	-
PromE>InR^{DN}	TOR/PI3K	-	↓	-	-	ND	-
PromE>P60	TOR/PI3K	-	↓	ND	-	ND	-
PromE>Hnf4 RNAi	Nuclear receptor	↑	-	-	↑	ND	-
PromE>Sam-S RNAi	One carbon metabolism	-	-	-	ND	ND	-
Lpp>bmm	Lipase	-	ND	ND	ND	ND	NA
Lpp>Lsd2	Lipid droplet protein	-	ND	ND	ND	ND	NA
Lpp>SREBP^{DN}	Lipid synthesis	+	ND	ND	ND	ND	NA

Figure 7.2 Speculative model for adult oenocyte function

In this model, lipid droplets (red) decrease in the fat body but accumulate in oenocytes with age. The oenocytes, fat body and gut grow in a nutrient dependent manner during adulthood, with the TOR/PI3K signalling pathway required for oenocyte growth. Dietary methionine deficiency promotes lipid droplet accumulation in oenocytes, suggesting that they may be regulated in a similar manner to mammalian hepatocytes. LpR1-mediated lipid uptake from the haemolymph occurs on both control and methionine-deficient diets. Lipids present in oenocytes can be channelled towards Cyp4g1/cpr-mediated cuticular hydrocarbon synthesis. An oenocyte-derived signal promotes fat body lipolysis. In addition, inhibiting lipid synthesis in the fat body promotes oenocyte lipid droplet accumulation. One possible explanation for this effect is that both the oenocytes and fat body compete for *de novo* lipogenesis precursors, such that reducing fatty acid synthesis in the fat body leaves more precursors for lipid synthesis in the oenocytes. Together, these results demonstrate that bidirectional metabolic coupling exists between the oenocytes and fat body. The possibility is also raised that adult oenocytes may regulate midgut lipid metabolism.


Dietary sugar/amino acids
Gut
Fat body
Lpp
?
Lpp
?
InR
LpR1
ER
Elongase
Cyp4g1/cpr
LCFA
HC
FA
Nucleus
Hnf4
β oxidation
Oenocyte
Cuticle
YOUNG ADULT FLY
PI3K/TOR
Dietary sugar/amino acids
Lpp
?
Lpp
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InR
LpR1
ER
Elongase
Cyp4g1/cpr
LCFA
HC
FA
Nucleus
Hnf4
β oxidation
OLD ADULT FLY

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APPENDIX

CHEMICALLY DEFINED DIET FLY FOOD No.22

Dist Water	0.7500	l	
Sucrose	10.0000	gm	
L-Alanine	0.5000	gm	
L-Arginine	0.8000	gm	
L-Aspartic Acid	0.5000	gm	
L-Cysteine	0.5000	gm	
L-Glutamic Acid	5.4000	gm	
Glycine	0.5000	gm	
L-Histidine	1.0000	gm	
L-Isoleucine	3.0000	gm	
L-Leucine	2.0000	gm	
L-Lysine HCl	1.9000	gm	
L-Methionine	0.8000	gm	
L-Phenylalanine	1.3000	gm	
L-Proline	0.5000	gm	
L-Serine	0.5000	gm	
L-Threonine	2.0000	gm	
L-Tryptophan	0.5000	gm	
L-Tyrosine (dissolve in KOH)	0.5000	gm	
L-Valine	2.8000	gm	
Cholesterol	0.2000	gm	
Adenosine-5'(3')-monophosphate	0.6000	gm	
Guanosine-5'(3')-monophosphate	0.4000	gm	
Uridine-5'(3')-monophosphate	0.4000	gm	
Cytidine-5'(3')-monophosphate	0.4000	gm	
NaHCO3	1.0000	gm	
KH2PO4	0.7100	gm	
K2HPO4	3.7330	gm	
MgSO4.7H2O	0.8200	gm	
NaCl	0.0400	gm	
Fe.Na EDTA	0.0200	gm	
Zn.Na EDTA	0.0200	gm	
Mn.NA EDTA	0.0200	gm	
Cu.Na EDTA	0.0050	gm	
Nipagin/Bavistan Solution	19.6400	ml	
Adjust pH with HCl to	6.5000		
Agarose (Low Melting Point)	10.0000	gm	Cat No. A9414
Bring to the boil			
Allow to cool to 43°C			

Dissolve Cholesterol in 19.64ml Nip/Bav Soln.		
Choline Chloride	0.0600	gm
Ca Gluconate	0.0500	gm
Thymidine	0.2000	gm
Thiamine HCl	0.0020	gm
Riboflavin	0.0100	gm
Nicotinic Acid	0.0120	gm
Ca Pantothenate (DL used)	0.0160	gm
Pyridoxine HCl	0.0025	gm
Folic Acid	0.0030	gm
DL-Carnitine HCl	0.0100	gm
Biotin	0.0002	gm
Penicillin	0.2500	gm
Streptomycin	0.2500	gm
Dist Water	0.2500	l
Adjust pH with Conc.HCl to	6.5000	
Filter Sterilize		
Allow both solutions to cool below 43°C before mixing (as cool as possible, without setting)		
Aliquote in 13ml amounts into Vials		

CHEMICALLY DEFINED DIET FLY FOOD No.24

Dist Water	0.7500	l	
Sucrose	10.0000	gm	
Tryptone	55.0000	gm	
Cholesterol	0.2000	gm	
Adenosine-5'(3')-monophosphate	0.6000	gm	
Guanosine-5'(3')-monophosphate	0.4000	gm	
Uridine-5'(3')-monophosphate	0.4000	gm	
Cytidine-5'(3')-monophosphate	0.4000	gm	
NaHCO3	1.0000	gm	
KH2PO4	0.7100	gm	
K2HPO4	3.7330	gm	
MgSO4.7H2O	0.8200	gm	
NaCl	0.0400	gm	
Fe.Na EDTA	0.0200	gm	
Zn.Na EDTA	0.0200	gm	
Mn.NA EDTA	0.0200	gm	
Cu.Na EDTA	0.0050	gm	
Nipagin/Bavistan Solution	19.6400	ml	
Adjust pH with HCl to	6.5000		
Agarose (Low Melting Point)	10.0000	gm	Cat No. A9414
Bring to the boil			
Allow to cool to 43°C			

Dissolve Cholesterol in 19.64ml Nip/Bav Soln.		
Choline Chloride	0.0600	gm
Ca Gluconate	0.0500	gm
Thymidine	0.2000	gm
Thiamine HCl	0.0020	gm
Riboflavin	0.0100	gm
Nicotinic Acid	0.0120	gm
Ca Pantothenate (DL used)	0.0160	gm
Pyridoxine HCl	0.0025	gm
Folic Acid	0.0030	gm
DL-Carnitine HCl	0.0100	gm
Biotin	0.0002	gm
Penicillin	0.2500	gm
Streptomycin	0.2500	gm
Dist Water	0.2500	l
Adjust pH with Conc.HCl to	6.5000	
Filter Sterilize		
Allow both solutions to cool below 43°C before mixing (as cool as possible, without setting)		
Aliquote in 13ml amounts into Vials		